How insects learn about different goal locations:

An analysis of learning and return flights of male and worker bumblebees at the nest and at a feeding site

Submitted by Theo Geoffrey ROBERT to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Psychology

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature……..

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Thesis summary

Bees and wasps perform learning flights when departing their nest for the first few times or a newly discovered food source. Several studies have described the occurances and structure of these flights in several species, but few have examined how the insects systematically vary the characteristics of their learning flights in various conditions in order to aid the acquisition of visual information. This is best done in a species where individuals and nests can be easily manipulated and tested repeatedly. The aim of this thesis was therefore to investigate learning flights in bumblebees, where we have a good understanding of the structure and variability of flights from previous work and can design controlled experiments. I explored the similarities and differences of learning flights of workers and male bumblebees, observing their departures from the nest or an artificial flower. A second objective was to examine how differences in the learning flights affect the bumblebees' ability to return the learnt location. The experiments were conducted inside a large greenhouse, under natural light regimes, with two large tables placed far apart, one for simulating the ground from which bees emerged when departing their nest, and the other representing a feeding site with an artificial flower.

Female bumblebees performed shorter learning flights when leaving a flower than when leaving their nest, although both locations displayed similar visual scenes. At both locations, the duration and trajectory length of learning flights decreased over successive visits, but the decrease was faster at the flower location than at the nest. Bumblebees fixated both their nest and the flower during their learning flights as well as the landmarks available around the two locations, which suggests that they learned the position of the goal relative to these landmarks.

When the nest and the flower were hidden and only three cylinders were shown as landmarks in tests, bees searched as accurately for the nest as for the flower. However, they were more persistent when searching for the nest than for the flower, which was not predicted from the variation of learning flights at the nest and flower locations. Another situation in which bumblebees varied the characteristics of their learning flights, but without an impact on their performance when recalling the learnt information, was after visiting flowers filled with low and high sucrose rewards. The bees performed longer learning flights after drinking at a highly rewarded flower. When departing a poorly rewarded flower, bumblebees did not fixate the flower during their learning flights. Nevertheless, the bees were able to return to both the poorly rewarded flower and the highly rewarded flower equally fast. Given the above findings, it is not evident how different durations or trajectory lengths of bumblebee learning flights might be linked to variations in learning of goal locations.

Finally, I show that bumblebees of either sex decide to perform learning flights at locations that are of importance to them. Whilst the female workers always performed learning flights when departing their natal nest, the males did not and simply flew away in a straight line. However, when leaving a flower, the males did perform learning flights with characteristics similar to those of the females' learning flights. They were also able to return to the flower, showing similar approach trajectories as workers.

The thesis discusses these findings in the light of ideas and hypotheses that are linked to differential investment in learning which were observed in the various conditions here. It is also discussed why bumblebees used fixations in different ways when learning about the visual environment surrounding goals that are important to them. Whilst many results are parsimonious with the requirements for learning and active flight control to aid the acquisition of visual information, motivation also seems to play a role in varying the occurances and features of learning flights, such as seen in the bees' greater persistence to search for their nest than for a flower.

Declaration

The work reported in this thesis has involved collaboration with Elisa Frasnelli (EF) and Ben Scales (BS). Their contributions are listed below. As my supervisors, Natalie Hempel de Ibarra (NH) and Tom Collett (TS) were involved in all chapters and provided comments on the write up.

Chapter 2: EF assisted in data collection, extracted flight trajectories from a subset of videos and participated in discussions about the design of the experiments. The work is in preparation for publication.

Chapter 3: The included data were collected and video analysis conducted by EF and BS. EF performed some exploratory data analysis and participated in discussions about the design of the experiments.

Chapter 4 has been published in the Journal of Experimental Biology:
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Figure and table captions

Figure caption

Chapter 1

Figure 1.1. Distribution of the fixation lengths (A and B), distribution of inter-fixation lengths (C and D) and distribution of flights relative to their proportion used in performing fixations (E and F) using the parameters 3° maximum body orientation variation and 4 frames minimum fixation length (left column) and for 5° maximum body orientation variation and 4 frames minimum fixation length (right column). These graphs combine the learning and return flights 1 to 4 recorded during the experiment presented in Chapter 2.

Chapter 2

Figure 2.1. Schematic representation of our experimental setup during the second experiment. The nest hole was surrounded by a black-white patterned ring, whilst the inconspicuous flower was surrounded by three cylinders.

Figure 2.2. Median durations of learning flights when departing the nest or inconspicuous flower. In Experiment 1 the nest and flower were surrounded by three cylinders. In Experiment 2 only the flower was surrounded by the same three cylinders, whilst the nest hole was marked by a ring with a black-white radial pattern.

Figure 2.3. Satellite view of the greenhouse where the experiments were conducted. The red arrow indicates the real North direction. The experimental tables were set at the southern end of the West greenhouse. (©Goggle Earth)

Figure 2.4. Picture of the experimental tables set up in the greenhouse. In the foreground is the nest table and in the background is the flower table. The black cylinders are visible on each table as well as the purple plastic rings marking the position of each location.

Figure 2.5. Schematic of the last foraging trips showing the order of the test without and with the purple plastic ring placed on the experimental tables. The rectangles represent top view of the flower table (left column) and nest table (right column). The black circles represent the three cylinders. The purple circles indicate that the purple ring was on the table during the bees' approaches. The nature of the return is indicated at the top right corner of the tables. During the "Normal returns", the bees could access their nest or feed on the flower. During the "Tests", the nest entrance was hidden and the flower was unrewarded. The black arrow indicates the direction of the real North.

Figure 2.6. On the left: Top view of the trajectory of the bee FB4 during her first departure from the nest (A) and the flower (B), and the fourth departure from the nest (C) and the flower (D). The large black filled circles represent the position of the cylinders. The green circle shows the location of the nest or the flower. The empty circles are the positions of the bee plotted every two frames and the tails represent the orientation of the bee. The blue circles represent the frames on which the bee is oriented toward the nest or the flower (± 10°). On the right: Bees' cardinal orientation, orientation relative to nest or flower, retinal position of the nest or flower and distance from the nest or flower during the learning flights. Insets: Illustrations of

the angles corresponding to the body orientation relative to the line between the flower and central landmark (F-C) (θ), angular position (β) and the position of the nest or the flower relative to the bee's longitudinal axis (ϕ, 'Retinal' position for short). Green circles indicate the nest or the flower. The arrows points in the positive direction.

Figure 2.7. A: Median times taken by the bees before to cross different distances from the nest or the flower during the learning flights 1 to 4 at the nest (white symbols) and at the flower location (black symbols). The times are measured before the bees crossed a circle of a given radius to a maximum of 24 cm. B: Medians of each bee's mean speed when crossing different distances from the nest or the flower for the first time ± 2 frames. C: Median trajectory length flown by the bees before to cross different distances from the nest or the flower. D: Medians of the bees' median distances from the goal within each tenth of the learning flights. For each individual, the flight was divided in ten equal parts in duration and the bee's median distance from the goal was computed for each of these ten sections. The legend for the four graphs is shown in figure C. Here, and later in the thesis, the learning flights at the flower 1 to 4 are called LF01, LF02, LF03 and LF04. The learning flights at the nest 1 to 4 are called LN01, LN02, LN03 and LN04.

Figure 2.8. Frequency distribution of the bees' flight directions relative to array-direction for the early part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°.

Figure 2.9. Frequency distribution of the bees' flight directions relative to array-direction for the late part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°.

Figure 2.10. Frequency distribution of the bees' flight directions relative to nest or flower for the early part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°.

Figure 2.11. Frequency distribution of the bees' flight directions relative to the nest or flower for the late part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°.

Figure 2.12. Heat maps of the kernel density estimations of the bees' position from the combined late phases of the first learning flights at the nest (A) and the flower (B). Each bee position was weighted in function of its time relative to the total duration of the flight (i.e. time of the frame/duration of the flight). The black circles represent the position of the cylinders and the cross is the position of the nest or the flower.

Figure 2.13. Frequency distribution of the bees' orientation relative to array-direction for the early part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°. Mean orientations and vector amplitude at the nest are: flight 1: mean=-49.98deg, rho=0.16; flight 2: mean=-36.27deg, rho=0.17; flight 3: mean=-79.30deg, rho= 0.39; flight 4: mean=-72.42deg, rho=0.16. Mean orientations and vector amplitude at the flower are: flight 1: mean=-

40.46deg, rho=0.38; flight 2: mean=-60.56deg, rho=0.16; flight 3: mean=132.61deg, rho= 0.11; flight 4: mean=119.56deg, rho=0.16.

Figure 2.14. Frequency distribution of the bees' orientation relative to array-direction for the late part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°. Mean orientations and vector amplitude at the nest are: flight 1: mean=5.04deg, rho=0.06; flight 2: mean=-25.10deg, rho=0.12; flight 3: mean=-1.67deg, rho= 0.19; flight 4: mean=-34.49deg, rho=0.16. Mean orientations and vector amplitude at the flower are: flight 1: mean=-33.38deg, rho=0.19; flight 2: mean=59.17deg, rho=0.09; flight 3: mean=-166.09deg, rho= 0.17; flight 4: mean=142.89deg, rho=0.44.

Figure 2.15. Frequency distribution of the bees' orientation relative to nest or flower for the early part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Mean orientations and vector amplitude toward the nest are: flight 1: mean=-3.31deg, rho=0.70; flight 2: mean=-2.53deg, rho=0.70; flight 3: mean=-3.28deg, rho=0.72; flight 4: mean=4.75deg, rho=0.60. Mean orientations and vector amplitude towards the flower are: flight 1: mean=9.84deg, rho=0.17; flight 2: mean=10.34deg, rho=0.13; flight 3: mean=-112.33deg, rho=0.43; flight 4: mean=-178.02deg, rho=0.42. Bin width is 20°.

Figure 2.16. Frequency distribution of the bees' orientation relative to nest or flower for the late part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Mean orientations and vector amplitude towards the nest are: flight 1: mean=2.98deg, rho=0.39; flight 2: mean=-3.05deg,

rho=0.37; flight 3: mean=5.25deg, rho=0.40; flight 4: mean=-5.63deg, rho=0.29. Mean orientations and vector amplitude towards the flower are: flight 1: mean=9.84deg, rho=0.17; flight 2: mean=-88.93deg, rho=0.15; flight 3: mean=-146.67deg, rho=0.29; flight 4: mean=-171.85deg, rho=0.41. Bin width is 20°.

Figure 2.17. Frequency distribution of the bees' fixations orientation relative to array-direction during the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 40°.

Figure 2.18. Frequency distribution of the bees' fixations orientation relative to nest or flower during the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 40°.

Figure 2.19. Normalised distribution of the facing fixations (±20°) depending on their distance from nest or flower during the first (A), second (B), third (C) and fourth (D) learning flights. The bars show the proportion of the fixations oriented toward the nest (in grey) or the flower (in black) that fall into each 5 cm distance bin.

Figure 2.20. Mean rates of fixations centred on the goal (±20°) within regular section of the learning flights 1 to 4 at the nest (white symbols) or the flower (black symbols). For each bee, the number of fixations in each flight section is divided by the number of frames present in this flight section.

Figure 2.21. Number of fixations toward the real (coloured) and virtual (grey) cylinders (±10°) depending on their radial position around the nest during the first (A), second (B), third (C) and fourth learning flights (D) or around the flower during

the first (E), second (F), third (G) and fourth learning flights (H). The darker bars indicate the number of the fixations that are also toward the nest or the flower (±10°).

Figure 2.22. Bees' position during fixations toward the cylinders (±20°) during the first (A), second (B), third (C) and fourth (D) learning flights at the nest and the first (E), second (F), third (G) and fourth (H) learning flights at the flower location. Each of the points represent the position of a bee during the first frame of a fixation toward the left (in blue), central (in yellow), or right cylinder (in red). The corresponding cylinders' positions are shown by the large coloured circles. The nest or flower location is marked by a black cross.

Figure 2.23. Mean rates of fixations centred on the cylinders (±20°) within regular section of the first learning flights at the nest (A) or the flower (B). For each bee, the number of fixations in each flight section is divided by the number of frames present in this flight section.

Figure 2.24. On the left: Top view of the trajectory of the bee FB4 during her first return at the nest (A) as well as her first approach (B) and first return to the flower (C), and the fourth return to the nest (D) and the flower (E). The large black filled circles represent the position of the cylinders. The green circle shows the location of the nest or the flower. The empty circles are the positions of the bee plotted every two frames and the tails represent the orientation of the bee. The blue circles represent the frames on which the bee is oriented toward the nest or the flower (± 10°). On the right: Bees' cardinal orientation, orientation relative to nest or flower, retinal position of the nest or flower and distance from the nest or flower during the learning flights.

Figure 2.25. A: Median times taken by the bees before to cross different distances from the nest or the flower during the return flights 1 to 4 at the nest (white symbols) and at the flower location (black symbols), and the first approach of the flower (red symbols). The times are measured starting from the first crossing of the 26 cm distance to before the bees crossed a circle of a given radius to a minimum of 6 cm. B: Medians of each bee's mean speed when crossing different distances from the nest or the flower for the first time ± 2 frames. C: Median trajectory length flown by the bees before to cross different distances from the nest or the flower. Here, and later in the thesis, the first approach of the flower is called RF00, the return flights to the flower 1 to 4 are called RF01, RF02, RF03 and RF04. The return flights to the nest 1 to 4 are called RN01, RN02, RN03 and RN04.

Figure 2.26. Frequency distribution of the bees' flight directions relative to orientation of the cylinder array for the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience (E). Mean direction to nest: return 1: mean=30.6deg, rho=0.23; return 2: mean=35.2deg, rho=0.35; return 3: mean=30deg, rho=0.30; return 4: mean=10.9deg, rho=0.25. Mean direction to flower: return 0: mean=-1.52deg, rho=0.51; return 1: mean=8.32deg, rho=0.50; return 2: mean=2.99deg, rho=0.60; return 3: mean=-10.8deg, rho=0.58; return 4: mean=-7.06deg, rho=0.64. Bin width is 20°.

Figure 2.27. Frequency distribution of the bees' flight directions relative to the goal for the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience (E) Direction relative to nest: return 1: mean=3.6deg, rho=0.42; return 2: mean=-2.17deg,

rho=0.61; return 3: mean=-3.09deg, rho=0.50 ; return 4: mean=-0.69deg, rho=0.58. Direction relative to flower: return 0: mean=-0.91deg, rho=0.61; return 1: mean=-4.99deg, rho=0.72; return 2: mean=1.99deg, rho=0.78; return 3: mean=-4.83deg, rho=0.75; return 4: mean=-6.84deg, rho=0.79. Bin width is 20°.

Figure 2.28. Frequency distribution of the bees' orientations relative to array-orientation for the first (A), second (B), third (C) and fourth (D) return flights to the nest (grey) or flower (black) location and the first approach of the flower prior to any experience (E). Approaches to flower: return 0: mean=2.22deg, rho=0.74; return 1: mean=-9.01deg, rho=0.64; return 2: mean=-5.81deg, rho=0.69; return 3: mean=13.89deg, rho=0.67; return 4: mean=12.06deg, rho=0.73. Approaches to nest: return 1: mean=-14.52deg, rho=0.33; return 2: mean=-20.11deg, rho=0.47; return 3: mean=-15.69deg, rho=0.38; return 4: mean=-12.09deg, rho=0.36. Bin width is 20°.

Figure 2.29. Frequency distribution of the bees' orientations relative to the goal for the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience (E). Approaches to flower: return 0: mean=1.95deg, rho=0.91; return 1: mean=-0.53deg, rho=0.94; return 2: mean=1.51deg, rho=0.94; return 3: mean=-0.08deg, rho=0.92; return 4: mean=-2.17deg, rho=0.90. Bin width is 20°.

Figure 2.30. Frequency distribution of the bees' fixations relative to array-orientation during the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience of the location (E). Approaches to flower: return 0: mean=10.19deg, rho=0.74; return 1: mean=-4.01deg, rho=0.79; return 2: mean=4.82deg, rho=0.77;

return 3: mean=17.72deg, rho=0.67; return 4: mean=-4.39deg, rho=0.77. Approaches to nest: return 1: mean=-40.88deg, rho=0.28; return2: mean=-14.99deg, rho=0.49; return 3: mean=-36.95deg, rho=0.36; return 4: mean=-0.44deg, rho=0.51 Bin width is 40°.

Figure 2.31. Frequency distribution of the bees' fixations relative to the goal during the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience of the location (E). Approaches to flower: return 0: mean=-3.46deg, rho=0.92; return 1: mean=-1.85deg, rho=0.92; return 2: mean=-8.10deg, rho=0.98; return 3: mean=-3.02deg, rho=0.94; return 4: mean=10.02deg, rho=0.92; Watson-Wheeler test across trial, W=12.97, p=0.11. Approaches to nest: return 1: mean=-6.23deg, rho=0.55; return 2: mean=1.68 deg, rho=0.87; return 3: mean=-12.19deg, rho=0.83; return 4: mean=-11.02deg, rho=0.89; Watson-Wheeler test across returns 1 to 4, W=19.1, p=0.004. Bin width is 40°.

Figure 2.32. Normalised distribution of the facing fixations (±20°) depending on their distance from nest or flower during the first (A), second (B), third (C) and fourth (D) return flights and the first approach of the flower prior to any experience of the location (E). Mean fixation direction and rho during returns to the flower: return 0: mean=-3.46deg, rho=0.92; return 1: mean=-1.85deg, rho=0.92; trial 2: mean=-8.10deg, rho=0.98; return 3: mean=-3.02deg, rho=0.94; return 4: mean=10.02deg, rho=0.92; Watson-Wheeler test across trial, W=12.97, p=0.11. Mean fixation direction and rho during returns to the nest: return 1: mean=-6.23deg, rho=0.55; return 2: mean=1.68deg, rho=0.87; return 3: mean=-12.19deg, rho=0.83; return 4:

mean=-11.02deg, rho=0.89; Watson-Wheeler test across returns 1 to 4, W=19.1, p=0.004. The bars show the proportion of the fixations oriented toward the nest (in grey) or the flower (in black) that fall into each 5cm distance bin.

Figure 2.33. Mean rates of fixations centred on the goal (±20°) before to cross different distances from the nest (white symbols) or the flower (black symbols) during the return flights 1 to 4 and the first approach of the flower prior to any experience of the location (red symbols). For each bee, the number of fixations in each flight section is divided by the number of frames present in this flight section.

Figure 2.34. Number of fixations toward the real (coloured) and virtual (grey) cylinders (±10°) depending on their radial position around the nest during the first (A), second (B), third (C) and fourth return flights (D) or around the flower during the first (E), second (F), third (G) and fourth return flights (H) and the first approach of the flower prior to any experience of the location (I). The darker bars indicate the number of the fixations that are also toward the nest or the flower (±10°).

Figure 2.35. Bees' position during fixations toward the cylinders (±20°) during the first (A), second (B), third (C) and fourth (D) return flights at the nest and the first (E), second (F), third (G) and fourth (H) return flights at the flower location and the first approach of the flower prior to any experience of the location (I). Each of the points represent the position of a bee during the first frame of a fixation toward the left (in blue), central (in yellow), or right cylinder (in red). The corresponding cylinders' positions are shown by the large coloured circles. The nest or flower location is marked by a black cross.

Figure 2.36. Positions of the first landing (green crosses), landings after approaches (blue crosses), repeated landings (grey crosses) and at the cylinders (orange crosses). The virtual position of the goals relative to the cylinders are marked by empty black circles for the tests without purple ring at the nest (A) and the flower location (B). The same goals locations are shown by purple circles for the tests with the rings placed on the nest (C) and flower (D) tables. The cylinders' positions are shown by the large black filled circles.

Figure 2.37. Normalised distributions of the landings following approaches depending on their distance from the virtual position of the goals computed relative to the position of the cylinders for the tests without rings (A) and with rings (B) at the nest (grey) and flower location (Black). The same plots including the repeated landing are shown for the test without the rings (C) and with the rings (D).

Figure 2.38. Examples of trajectories during the early search bouts of the tests at the nest location without (A) and with (B) purple ring and at the flower locations without (C) and with the rings (D). The large black filled circles represent the position of the cylinders. The purple circles show the virtual locations of the nest or the flower computed relative to the position of the cylinders. The empty circles are the positions of the bee plotted for frames and the tails represent the orientation of the bee. In green are the three slow down locations detected by our code.

Figure 2.39. Positions of the slow down points (green crosses) during the early search bouts. The virtual position of the goals relative to the cylinders are marked by empty

black circles for the tests without purple ring at the nest (A) and the flower location (B). The same goals locations are shown by purple circles for the tests with the rings placed on the nest (C) and flower (D) tables. The cylinders' positions are shown by the black filled circles.

Figure 2.40. Normalised distributions of the slow down points depending their distance from the virtual position of the goals computed relative to the position of the cylinders for the tests without rings (A) and with rings (B) at the nest (grey) and flower location (Black).

Figure 2.41. Frequency distribution of the bees' orientations relative to the virtual position of the nest (in grey) or the flower (in black) during their slow down points for the tests without (A) and with (B) the rings on the experimental tables. Bin width is 20°.

Figure 2.42. Frequency distribution of the bees' orientations relative to array direction during their slow down points for the tests without (A) and with (B) the rings on the experimental tables at the nest (in grey) and flower (in black) locations. Bin width is 20°.

Figure 2.43. Frequency distribution of the bees' orientations relative to the left cylinder during their slow down points for the tests without (A) and with (B) the rings on the experimental tables at the nest (in grey) and flower (in black) locations. Bin width is 20°.

Figure 2.44. Frequency distribution of the bees' orientations relative to the central cylinder during their slow down points for the tests without (A) and with (B) the rings on the experimental tables at the nest (in grey) and flower (in black) locations. Bin width is 20°.

Figure 2.45. Frequency distribution of the bees' orientations relative to the right cylinder during their slow down points for the tests without (A) and with (B) the rings on the experimental tables at the nest (in grey) and flower (in black) locations. Bin width is 20°.

Chapter 3

Figure 3.1. Volume drunk and drinking time when inexperienced bumblebees encounter a flower for the first time. (A) The bees' drinking time is correlated with the volume that they drink (20%: ρ=0.66, n=23, P=0.001; 50 %: ρ=0.74, n=18, P<0.0001) and both drinking volume (20%: median 37.46, IQR=27.74; 50%: median 55.90, IQR=35.22, t(39)=2.969, p=0.005) and drinking time (20%: median 46.36, IQR=18.84; 50%: median 63.47, IQR=17.05, t(39)=3.340, p=0.002) are greater for bees given 50% sucrose than for those given 20% sucrose. (B) Bees drank larger volumes when rewarded with 50% than with 20% (Median (20% volume drunk)=36.6µl, IQR=27.13; Median (50% volume drunk)=62.66µl, IQR=39.48; Mann-Whitney U=84, Z=-3.23, p=0.001).

Figure 3.2. Durations of learning flights when departing a flower. A different group of bees fed at flowers of one of four concentrations (10%, 20%, 30%, or 50% w/w). A

one way ANOVA shows a significant difference across all groups $F(3)= 4.893$; $p= 0.003$. The duration of the learning flights on leaving the flower differed significantly between the three lower (10%, 20%, 30%) and the two higher concentrations (30%, 50%), but not within the two lower or two higher concentrations (post-hoc Tukey HSD, $p<0.05$).

Figure 3.3 Selected examples of learning flights. Each point depicts the position of the bee at every second frame, i.e. every 0.04s. The line indicates the body orientation of the bee. The location of the flat pink artificial flower (5cm in diameter) is shown as a green dot. The three black dots represent the three black cylinder surrounding the flower. The concentration of the reward imbibed prior to the learning flight (percentage, w/w) and the duration of the learning flight are shown for each example.

Figure 3.4. Effects on learning flights of different sucrose concentrations on the flights' duration, trajectory length and the bees' distance from the nest. (A, B) Cumulative median flight durations and trajectory lengths before bees first cross successive radial distances from the flower increasing in steps of 2cm to a maximum of 24 cm. (One way ANOVA $F(2)=7.107$, $p=0.003$) shows that duration of the flights increased with concentration. Post-hoc Tukey HSD shows significant differences between 20% and 30% ($P=0.017$) and between 20% and 50% ($P=0.001$). The trajectory lengths did not vary significantly with concentration (one way ANOVA $F(2)=2.395$, $p=0.110$). (C) Median distance from nest over the course of learning flights. Each flight to a maximum distance of 24 cm is divided into 10ths and a bee's median distance from the flower calculated over each 10th. Plot shows that these

normalised distances are very similar for different concentrations (GEE χ^2=1.81, df=2, p=0.41).

Figure 3.5. Properties of fixations relative to the flower associated with different sucrose concentration. Frequency distributions of fixations relative to flower. After drinking 50% sucrose the distribution of fixations during the subsequent learning flight is centred on zero (mode= 0^o, circular variance=0.865). At lower concentrations, the mode is less prominent and the distributions have greater variances although, although the distribution of the 20% and 30% concentrations are not significantly different (30%: mode=-40^o, circular variance=0.993; 20%: mode=-40^o, circular variance=0.874; Watson-Wheeler test 20%vs30%: W=6, df=2, p=0.05; Watson-Wheeler test 30%vs50%: W=5, df=2, p=0.09; Watson-Wheeler test 20%vs50%: W=20, df=2, p<0.001).

Figure 3.6. Number of fixations as a function of the bees' distance from the centre of the flower. For all concentrations, the majority of is within 10 cm of the flower. The preponderance is greater for the two higher concentrations but not significantly different (Kruskal-Wallis, $\chi^2(2)$=6, p=0.06).

Figure 3.7. Rates of fixations relative to the flower associated with different sucrose concentrations. (A) Rate of fixations of the flower (±20^o) are plotted against relative time during the flights. Fixation rates after drinking 50% and 30% sucrose peak within the first third of the flight. Fixation rates for 20% are low throughout the flight. (B) Rate of fixations in which the bees' body is oriented more than 20^o from the flower (C, D) As A and B but fixation rate is plotted relative to distance from centre

of flower. Rates of fixations of flower after drinking 50% and 30% sucrose peak at 6 cm from flower. The residue of fixations (D) have no clear peaks for any concentration.

Figure 3.8. First return flights to flowers containing different sucrose concentrations**.** (A, B). Trajectory length and duration plotted as in Figure 3. There is no significant difference between returns to sucrose concentrations of 20% (N=10) and 50% (N=16). (Duration: Mann-Whitney, U=60, z=-1.78, p=0.08; Trajectory length: Mann-Whitney, U=70, z=-1.53, p=0.1). (C) Rates of fixation relative to the flower. The distributions for both sucrose concentrations peak when bee faces flower (20%: mode=0, circular variance=0.911; 50%: mode=0, circular variance=0.724; Watson-Wheeler test: W=1, df=2, p=0.5).

Figure 3.9. Drinking and learning flight durations when sucrose concentration is raised during a sequence of foraging trips for 13 bees that completed the whole experiment**.** (A) Drinking time during three visits to a flower when it contained 20% sucrose followed by two further flights after the concentration was raised to 50%. Drinking time rises significantly between learning flights 1 to 3 and 4 to 5 (one way ANOVA 20%-50% F(1)=12.586, p=0.001). (B) Duration of learning flights after drinking. Duration of the flights drops significantly between flight 1 to 3 and flights 4 to 5 (one way ANOVA F(1)=5.022, p=0.029).

Chapter 4

Figure4.1. Experimental setup in a greenhouse: nest and flower tables. The nest table

is in the foreground with the nest fixed under the table and its exit through a hole near the array of three cylinders. The flower table is in the background with the artificial flower in the same position relative to the cylinders as at the nest hole.

Figure 4.2. Example trajectories of male bumblebees. (A) Departure from the nest. (B) Departure from the flower. Flights in A and B are by the same male. (C) Departure from the flower by another bee after its spontaneous return to the flower. (D) Enlarged view of the initial segment of the flight in B. Red circles indicate fixations of the flower (see Materials and methods). Blue squares mark every 0.5 s from the start of the flight. (E) Return to the flower. In all panels except D, each dot shows the position of the bee every 0.04 s and each line shows the orientation of the bee's body. Red circles and lines indicate instances in which the bee faced the flower. Positions of the nest and flower are shown by a green circle. Black circles represent the array of three cylinders.

Figure 4.3. Further example trajectories of a male (A) and worker (B) leaving the flower. In some learning flights bees turn for long periods in the same direction, rotating clock- or counter-clockwise through several complete revolutions. In other flights bees alternate their direction of rotation. Each dot depicts the position of the bee every 40 ms. The line shows the bee's body orientation. Red circles and lines indicate when the bee faces the flower. Green circle shows the position of the flower. Time plots show the bee's cumulative angular position (β), its body orientation (θ) relative to the line between the flower and central landmark (F-C), and the position of the flower relative to the bee's longitudinal axis (ϕ, 'Retinal' position for

short). Arrows (inset) point in a positive direction. Moments in which the bee faced the flower (±10°) are shown in red.

Figure 4.4. Further similarities in the learning flights of a bumblebee male (A) and worker (B) leaving the flower. For details see Figure 4.3.

Figure 4.5. Analysis of flower fixations in male learning flights. (A,B) Distribution of the absolute values of angles between the male's body orientation and the line from bee to flower ($|\phi|$) for every frame in every flight (4872 frames, N=24 males, n=24 flights; A) and for every frame in every fixation (821 frames; B). (C,D) Distribution of the mean $|\phi|$ of each randomly picked sample of individual frames (C) and groups of frames (D). In both cases, 100,000 samples were randomly selected. The red lines show the measured means of body angle orientation during fixations. Their position outside the distributions of the means of the randomly selected frames and groups of frames demonstrates that the measured distribution of body orientations in fixations is not a random selection from the overall distribution of body orientations during learning flights of male bumblebees. Inset in B shows the angle ϕ between the flower (F, green circle) and the bee's longitudinal body axis.

Figure 4.6. Some properties of male and female learning flights. (A) Duration of male departure flights from the flower (i.e. time taken to cross a 30 cm radius circle around the flower), plotted against time spent drinking on the flower before departure (N=30 males). Filled circles represent males with a short drinking time (bottom quartile) that were excluded from further analysis (N=6 males). (B) Duration of male departure flights from the nest and flower until crossing the 24 cm radius. (C)

Duration of female departure flights from the nest and flower until crossing the 24 cm radius. The flights of males from the flower were a little shorter (N=24 males, n=24 flights, mean±s.e.m. 3.58±0.54 s) than those of workers (N=14 workers, n=14 flights, mean±s.e.m. 4.94±0.72 s) (Mann–Whitney U-test, U=109, Z=−1.79, P=0.07). Worker flights from the nest lasted longer than the other three categories, i.e. male flights from the nest and flower and worker flights from the flower (14.42±1.20 s). (D) Cumulative trajectory lengths flown by males leaving the nest or flower and by workers leaving the flower. Lengths at different distances from the flower or nest are those measured before the bee first crossed a circle of a given radius to a maximum of 30 cm. The mean±s.e.m. total trajectory length of males leaving the nest was 40.25±2.74 cm and that of males leaving the flower was 98.53±12.05 cm (Wilcoxon, W=21, Z=−3.69, P=0.0002). The mean±s.e.m. trajectory length of workers leaving the flower was 90.75±13.25 cm. It was slightly but not significantly shorter than the trajectories of males leaving the flower (Mann–Whitney U-test, U=159, Z=−0.27, P=0.79). See also Figure 4.3 and 4.4.

Figure 4.7. Fixations of flower and nest during learning flights. (A) Frequency distribution of male body orientation relative to the nest (grey line, 1169 frames) or flower (black line, 4872 frames; N=24 males) on departures from them. Bin width is 20 deg. (B) Distribution of body orientation relative to the flower when all frames of male departure flights are partitioned into those within fixations (821 frames) and those outside fixations (4051 frames). (C) Frequency distribution of male and worker fixations (175 male fixations, 162 worker fixations; N=24 males, N=14 workers) relative to the flower (40 deg bin width). (D) Proportion of flights with at least one flower fixation (ϕ=0±20 deg) within a specified distance from the flower (N=10

males, N=9 workers). The numbers above each 5 cm bin give the total number of fixations falling in that bin. The lines above the histograms show the proportion of flights at each distance that are at least as long as that distance (N=24 males, N=14 workers).

Figure 4.8. Duration of fixations in male and female learning flights. Normalised distributions of the duration of all fixations in (A) males (n=175 fixations, N=24 males) and (B) workers (n=162 fixations, N=14 workers). Male and female fixations are partitioned into those in which bees faced the flower (ϕ=0±20 deg; males n=47 fixations, workers n=33 fixations) or did not face the flower (φ<−20 deg or φ>20 deg).

Figure 4.9. Comparison of male and worker returns to the flower. (A) Flight speed plotted against distance from the flower (N=8 males, N=14 workers). (B) Cumulative trajectory length as a function of distance from the flower. (C) Frequency distribution of body orientation relative to the flower (males, N=595 frames, workers N=1255). (D) Relative frequency of flower facing (ϕ=0±20 deg) plotted against distance from the flower (males, N=341 frames, workers N=811 frames). The lines above the histograms show the proportion of flights at each distance that are at least as long as that distance (N=8 males, N=14 workers).

Table captions

Chapter 2

Table 2.1. GEE modelling examining the influence of various predictors on several learning flights parameters. Entry 1: The flight duration decrease over trials while the learning flights at the nest were longer than the learning flights at the flower. Entry 2 and 3: The bees' mean ground speed when crossing radiuses of increasing distances from the goal. Bees' speed increased with their distance from the nest and the flower (entry 2). The bees were globally slower during their first flights (entry 2) but their speed did not significantly vary during the subsequent trials (entry 3). Entry 4: the trajectory length of the learning flights increased over trial and were longer at the nest than at the flower location. Entry 5: Bees' median distances from the nest or the flower within each tenth of the learning flights. The bees increased their distance from the two goals during their learning flights.

Table 2.2. GEE modelling examining the predictor influencing the bees' rates of fixation toward the goals (±20°) within each tenth of the learning flights (number of fixations in the flight section/number of frames in the flight section). Bees decreased their fixation rate during their flights as well as over trials. The learning flights at the nest presented higher fixation rated than the learning flight at the flower.

Table 2.3. GEE modelling examining the influence of various predictors on several return flights parameters. Entry 1: The flight duration did not vary over trials but the return flights at the nest were longer than the return flights at the flower. Entry 2: The bees' mean ground speed when crossing radiuses of decreasing distances from the

goal. Bees' speed decreased with their distance from the nest and the flower. The bees' ground speed did not vary over trial or between the two locations. Entry 3: The trajectory length of the return flights did not change over trial but were longer at the nest than at the flower location.

Table 2.4. GEE modelling examining the predictors influencing the bees' rates of fixation toward the goals (±20°) within each tenth of the return flights (number of fixations in the flight section/number of frames in the flight section). None of the predictors significantly influenced the fixation rates during the return flights.

Table 2.5. GEE modelling examining the effect of the presence or absence of the purple ring and the location on several indicators of the bees' search accuracy during the tests. Entry 1: The landings following approaches were more distant from the virtual positions of the goal when the ring was remove than when it was left on the table. They did not vary between locations. Entry 2: The landings following approaches and repeated were more distant from the virtual positions of the goal when the ring was remove than when it was left on the table. They did not vary between locations. Entry 3: The distances of the slowdowns from the virtual positions of the goal did not vary between the two locations and whether the ring was on the table or not.

Table 2.6. GEE modelling examining the effect of the presence or absence of the purple ring and the location on several indicators of the bees' search persistence during the tests. Entry 1: The rate of landings following approaches was higher when the ring was present than when it was removed and was higher at the nest location

than at the flower location, but the two effects were also in interaction. Entry 2: The rate of landings following approaches and repeated was higher when the ring was present than when it was removed and was higher at the nest location than at the flower location. Entry 3: The mean search bouts were longer when the ring was present than when it was removed and were longer at the nest location than at the flower location, but the two effects were, here again, in interaction.

CONTENTS

Chapter 1

Literature review

This chapter on hymnopteran behaviour deals with three broad topics that relate to the experimental chapters forming the bulk of the thesis. The first and most detailed sections are concerned with learning flights in bees and wasps, the second section with foraging behaviour and the third section with the way in which male bumblebees fit into the life of a bumblebee colony.

For animals, the ability to learn important locations in their environment provides a significant advantage on their fitness. They can either avoid dangerous locations or return to places of particular interest for them. Over the past 50 years, studies have explored how animals move between and learn different locations, even neurons coding for specific locations in the hippocampus of birds and mammals, the place cells, have been found (O'Keefe and Nadel, 1978). However, in insects, highly specialised brain structures dedicated to the processing of spatial information have not been fully uncovered yet (Wessnitzer and Webb, 2006). Despite their smaller and simpler brains, a wide range of insect families are able to navigate efficiently: Just like rats are capable of finding back a submerged platform in a Morris pool, crickets (Orthoptera, *Gryllidae*) and *Drosophila* (Diptera, *Drosophilidae*) can return to a cool spot location in a hot arena (Ofstad et al., 2011; Wessnitzer et al., 2008). Hoverflies (Diptera, *Syrphidae*), return to a precise place relative to the surrounding landmarks after chasing other insects (Collett and Land, 1975). Some aquatic beetles (Coleoptera, *Gyrinidae*) have been observed defending their territories, indicating their ability to recognise a specific location (Fitzgerald, 1987). Water striders (Hemiptera, *Gerridae*) maintain their position relative to a landmark where preys are frequent and return to this spot after small displacements (Junger, 1991) while some species of shield bugs (Hemiptera,

Parastrachiidae), return to their burrow to feed their larvae after foraging (Hironaka et al., 2008). Cockroaches (Blattodea, *Ectobiidae*) can find back their shelters (Rivault and Durier, 2004). Finally, termites' (Blattodea, *Termitidae*) foraging columns are able to extend as far as 300m from their nest and return safely to it (Jander and Daumer, 1974).Studies investigating insect navigation focus in their majority on Hymenopteran insects. The main reason for this particular interest most likely relates to the fact that Hymenopteran insects are central place foragers. They depart from the nest and explore a wide area to collect food. In order to return to their nest, they have to remember the location of the nest entrance. In 1882, Fabre already observed that some solitary bees released several kilometres away from their nest were able to find their way back to it (Fabre, 1882). Hymenopterans also memorise the locations of food sources in order to return to them repeatedly during several foraging trips.

But how do insects guide their path toward locations relevant to them? There are three main and not necessarily mutually exclusive homing strategies used by the different insects. The first one is based on chemotaxis and consists in following an odour plume or a pheromone trail. Following an odour gradient allows gregarious cockroaches to guide their final approach toward their shelter (Rivault and Durier, 2004). A similar process is also used by the ants *Cataglyphis* when they reached the vicinity of their nest (Steck et al., 2009; Buehlmann et al., 2012). There is also some evidence of the use of olfactory cues by bumblebees. Scented nectar appears to facilitate the detection of flowers by bumblebees (Heinrich, 1979). In other cases, bumblebees seem to mark rewarded flowers with a footprint pheromone encouraging their conspecific to land on the flower (Cameron, 1981). The use of pheromone trails is the main strategy used in several species of ants (Passera, 1984) and termites (Jander and Daumer, 1974) to guide their way from and back to their nest during their foraging activities.

A second navigation process is path integration (reviewed by Collett and Collett, 2000; Wehner, 2003; Srinivasan, 2015). It allows individuals to find their way in an unknown

environment. During travel from the nest the path integrator (PI) records the directions and distances of path segments and constantly calculates and updates the vector that will take the individual back to the nest. The PI involves two main biological systems: a compass to determine direction and an odometer to measure distance travelled. Path integration is used by a wide range of insects. For example, in honeybees this information is transmitted during the waggle dance to recruit foragers to a new food source. A dancing bee encodes directional and distance information about the food source she just visited as the direction relative to the sun and the flying distance from the hive (Riley et al., 2005). In desert ants, PI enables them to reach a foraging location in the absence of salient visual cues from landmarks or the panorama (Müller and Wehner, 1988; Wittlinger et al., 2006; Wittlinger et al., 2007). If the majority of the studies investigating path integration have been conducted on Hymenopterans, there is some empirical evidence of the use of this strategy by other insect orders such as cockroaches (Blattodea) (Durier and Rivault,1999; Rivault and Durier, 2004), crickets (Orthoptera) (Beugnon and Campan, 1989) or shield bugs (Hemiptera) (Hironaka et al., 2007; Hironaka et al., 2008).

Finally, the third main navigation strategy is view-based navigation. As for path integration, view-based navigation has been mostly studied in numerous Hymenopteran species such as ants, bees and wasps (Collett and Collett, 2002; Collett et al., 2013). However, this navigation technique is not limited to the hymenopterans as it has been shown in some species of flies (Diptera) (Collett and Land, 1975; Ofstad et al., 2011), crickets (Orthoptera) (Wessnitzer et al., 2008), cockroaches (Blattodea) (Rivault and Durier, 2004) and water strider (Hemiptera) (Junger, 1991).

1.1 View-based navigation in insects

In this thesis, I focus on learning flights, a special flight pattern observed when bees and wasps leave their nest for the first time. During learning flights these insects acquire visual information that allows them to form a memory of the spatial location of their nest. These view-based navigational memories are necessary to guide their final approach when returning to their nest or a newly discovered food source. I next discuss the current understanding about the functional significance of the structure of learning flights (section 1.1.1), that is, how it helps the insect to form and recall visual memories for their returns to their natal nest or a rich food source.

Since the classical experiments by Tinbergen (1932) on the digger wasp *Philanthus triangulum F*., it is known that visual landmarks near the nest are used by insects to pinpoint the exact location of their nest. He placed a circle of pine cones around a female's nest entrance just before the departure of the insect. He then displaced the pine cone circle while the wasp was hunting. When the wasp came back, it searched at the centre of the circle of pine cones showing that the wasp used the pinecones as cues to find her way back to her nest. This experiment also revealed the importance of the flight on departure for learning the disposition of pine cones. Later studies varied the size and location of landmarks placed around the nest entrance or a food source and have shown the insects' ability to distinguish the shapes, colours or brightness of the landmarks (reviewed by Collett and Collett, 2002).

The main mechanism of view-based navigation is image matching. Insects learn very quickly and become familiarised with visual features in their environment around the nest and the wider environment establishing routes along which they navigate between the nest and feeding locations. Image matching involves the formation of memories of views (also called

snapshots) at goal locations, such as the nest or a feeding location, and for views along routes. Memorised views are compared to current views, and the mismatch indicates the direction in which to move. Several mathematical models have captured how insects might convert such information into movement decisions (reviewed in Möller, 2012). Empirical evidence suggests that there are two fundamental ways, which can be distinguished as positional and alignment image matching according to Collett et al. (2013). For positional image matching, the insect uses a view recorded at one location to derive a direct path that will guide it directly to that location. Alignment image matching allows an insect to follow a visually guided route by turning so that its current view largely match a view previously stored along the route, but it does not necessarily compute a direct guidance path. Underpinning view-based navigation with the information derived from the PI gives insects a high degree of flexibility during navigation in familiar environments improving their ability to cope with disturbances and potential displacements on their routes.

1.1.1 Learning flights in hymenopteran insects

It is particularly important for bees and wasps to remember the location of their nest in order to return safely to it and provision the brood and colony members with food. Much of the essential information, as Tinbergen showed (1932), is acquired on the first departure from the nest. Numerous early studies have highlighted the elaborate structure of these first departure flights in bees and wasps (Fabre, 1882; Wagner, 1907; Wolf, 1926; Opfinger, 1931; Becker, 1958; Vollbehr, 1975). At a first glance these learning flights seem highly variable in their structure, but nevertheless they have some invariant features among and across species (reviewed by Zeil et al., 1996). I present, here, these common features and ask how they can help the insects to memorise the information needed during their returns.

In wasps and honeybees, a specific arcing pattern is often observed during the learning flights. Zeil (1993a) studied in detail the learning flights of two ground-nesting wasp species: *Cerceris rybyensis* and *Cerceris arenaria*. Placing a cylinder close to the nest entrances of these solitary wasps, induces learning flights composed of arcs of increasing radius centred on the nest hole and performed at a fairly stable angular speed.

Bees and wasps also perform learning flights when departing from a newly discovered food source. The learning flights at a food source have been studied in honeybees by Lehrer (Lehrer, 1991; 1993). When a bee takes off from a vertical feeder, it rotates its body up to 180° to face the food source. It then arcs and looks at it from several directions at close distance whilst slowly moving away from the food source. For this reason, Lehrer (1991) named this flight pattern the turn-back-and-look behaviour or TBL.

More detailed observations of the learning flights of insects leaving a feeder are available for the common wasp *Vespula vulgaris* (Collett and Lehrer, 1993; Collett, 1995). In this case an inconspicuous feeder was placed on the ground with its position indicated by nearby, vertical cylinder, the wasp's flew in arcing pattern Cerceris (Zeil, 1993a). It appears that arcing around the goal (e.g. a nest or a feeder) is a common characteristic of the learning flights of several species both at the nest and at a feeder location and that the pattern helps the insects to acquire important information about the visual environment. They generate a pattern of image centred on the goal termed 'pivoting parallax'. It provides an estimation of the landmark's distance relative to the goal (Zeil et al., 1996, Riabinina et al., 2014), as images in the visual field are displaced at a speed that decreases with the relevant objects' proximity to the goal. The animal may then use these estimations to apply a distance filter on the snapshot they memorised as suggested by Cartwright and Collett (1987) or simply determine which landmark is the most suitable to indicate the location of the goal.

Recently learning flights have been also studied in bumblebees (*Bombus terrestris*). In contrast with the arc patterns described in the wasps (Zeil, 1993a;1993b), the learning flights of bumblebees leaving their underground nest, are composed of loops (Philippides et al., 2013). During a brief part of these loops, the animal faces the nest and flies towards it, while significantly reducing its speed. The function of loops as opposed to arcs is probably to bring the bee all or part of the way to the nest and may be an adaptation to the low vegetation that obscures the bee's line of sight.

Some bees and wasps seem to face the nest or feeder at particular points of their learning flights. Vespula does so at the end of arcs while memorising snapshots during their learning flights (Collett and Lehrer, 1993; Collett, 1995). *Bombus terrestris* does so partway though most loops (Philippides, 2014). It is supposed that at these moments the insects may acquire views (or movies) of the nest or feeder and their setting. Zeil (1993a) suggested something different for *Cerceris sp.* He noted that during an arc the wasp maintains the nest entrance between 30 and 70° at the left or right of its body axis (depending on the arc direction), thus keeping the nest in the fronto-lateral eye region. In his review, Zeil and colleagues (1996) suggested that such a particular orientation keeps the frontal visual field, which has the highest resolution, available to scan the panorama around the goal. Zeil et al (1996) also proposed that the wasp memorised snapshots during its arcs and associated these snapshots with the local home vector. The totality of the vectors memorised would, then, point roughly at 45° either at the right or the left of the animal's body axis. This would facilitate homing by keeping homing wasps within the borders of the arcs. An alternative account of the particular orientations observed by Zeil (1993a) in *Cerceris* is that the arcs are involved in assessing distance or it could be a way for the wasp to pre-position itself in order to perform a subsequent stereotyped manoeuvre that is required to reach the correct position to face the goal and memorise a snapshot.

A role for such stereotyped manoeuvres have been proposed by Collett et al. (2013b) when discussing the presence of compass-oriented straight segments in the bumblebees' learning and return flights. During a straight segment, the flight direction is predominantly in one of few specific compass directions (N-E, N-W, S-W and S-E). These segments happen mostly at the beginning and the end of the loops or zigzags. The flight directions do not necessarily coincide with the body orientations and might be maintained using compass cues. If one assumes that these flight directions are determined at the beginning of a loop or a zigzag and combined with stereotyped flight control manoeuvres, this could represent a simple mechanism. This mechanism would help the insect to reach the correct orientation at the part of the loop when it faces and flies towards the nest, with less reliance on compass or visual cues to control this flight pattern.

Both learning flights at the feeder and at the nest share the same function – to memorise the views around significant goals to which a foraging bees wants to return. The flight of a returning bee differs from the learning flight but during a return flight, bees will adopt positions and orientations that are similar to those in their respective learning flights. Zeil (1993b) noticed that on return flights at the nest, *Cerceris sp.* wasps reproduce a similar arc pattern to that of its learning flight. The wasps, also as in their learning flights, are oriented in a compass direction that places the nest hole (which cannot be seen) in front of the landmark. Similar relations between learning and return flights occur in *Vespula vulgaris.* Collett (1995) compared the body orientation of the wasp facing the feeder on departure with the orientations assumed on return. The wasp faces the cylinder near to the feeder until it enters a 7 cm radius around the feeder, suggesting that the animal learnt that the landmark is a good indicator of the feeder place. Within 7cm around the goal, the insect's orientation becomes remarkably similar to the one it assumed when it was facing the food source during its departure. Also, in *Bombus terrestris*, similar nest facing parts are observed during the zigzag

shaped return flight. In fact, as in the loops, the bumblebee faces the nest and flies toward it roughly at 2/3 through a zigzag (Philippides et al., 2013). This coincidence of orientations between learning and the return flights when facing the nest can be interpreted as attempts to match their current view with previously memorised snapshots, strengthening the view a major function of learning flights is to acquire a series of snapshots (or movies) that the insects will follow during their return to the nest or flower.

In sum, learning flights seem to be used by the flying hymenopterans to estimate the distance of the landmarks relative to the goal and store snapshots needed to pinpoint the location of the goal on returns to it. These learning flights are underpinned by stereotyped flight manoeuvres, which nevertheless allow sufficient flexibility to produce variable flights and to adjust the structure of the flight facilitating the acquisition of views with different constellations of landmarks.

1.1.2 Learning on approach and departure

In some of the experiments presented in the following chapters, it was attempted to retain some ecological validity in the design of the experimental setup such that it would resemble the bees' natural foraging environment better. For this reason, we allowed bumblebees to freely depart from and return to their nest or to an artificial flower, but this also means that they had potentially more opportunities to relearn or consolidate their memory. Initially, it was thought that the hymenopterans learn different cues marking the presence of a point of interest either only during their arrival to it (Opfinger, 1931) or only on their departure from it (Gould, 1988). Gould trained honeybees with different sets of landmarks placed near a feeder so that they saw one set on arrival and another on departure. After a training phase, bees were given a choice between two empty feeders. One feeder displayed the landmarks seen on approach, the other those seen on departure. The insects chose significantly more the landmarks presented during their departure and Gould (1988) concluded that the bees learn the colour or shape of the landmarks only during their learning flights.

However, several studies have contradicted this conclusion. In 1993, Lehrer decomposed Gould's (1988) protocol by showing a landmark only during the bees' arrival or the departure and testing them by making them choose between the familiar landmark and a new one. It appeared that the honeybees were able to learn the colour, the shape and the relative location of a landmark during their arrival as well as during their departure. The bees learnt these landmarks characteristics significantly better if they could see them both on arrival and departure (Lehrer, 1993). A second study showed that the honeybees learn the apparent size of the landmarks near a feeder both during the approach and the departure, but it seems that the bees learn the distances of the landmarks relative to the feeder only during their learning flights (Lehrer and Collett, 1994).

Wei et al. (2009) demonstrated that if honeybees during training are displaced repeatedly while on a feeder from the place where they land to another location, they can learn to return to the location on which they landed as well as the one from which they departed (Wei and Dyer, 2009). This study confirmed that the honeybees can learn enough of the characteristics of the panoramic cues on the approach to a food source to be able to return to the location at which they landed. In light of this finding, it is reasonable to assume that the bumblebees are also able to learn the nest or a feeder location relative to landmarks during their returns. It can be therefore varied in experimental procedures, whether a bee is allowed to conduct returns to the nest or a feeding site, or not, with the latter precluding any learning on return. Instead bees can be either deprived of the return experience or tested upon return. The latter can be useful to measure the strength of the bee's memory and identify potentially different strategies of memory recall for localising the nest or feeding site.

The duration of learning decreases significantly every time honeybees leave the same food source until learning flights and turning back behaviours disappear completely (Lehrer, 1993). The insect then leaves the nest or feeder location in a more or less straight line, accelerating very quickly. During such departure the insect will not notice any changes in the visual environment, but only attends to them if it cannot localise the nest on its return.

The sequence of consecutive learning flights that an insect will perform at a single location is known in the literature as the "learning phase" (Lehrer, 1993). If honeybees are unsuccessful in finding the food source at a known location this can result in a temporarily increased duration of the next learning flights (Wei et al., 2002).

The duration of the learning phase and the length of the learning flights are also sensitive to changes in the value of a goal. Wei et al. (2002) increased the concentration of the sucrose solution offered to the bees at a familiar feeder that they previously visited, successfully restoring the length of the learning flights when the bees departed from this feeder. They also

showed that learning flight duration can depend on the complexity of visual scenes. When displacing the landmarks at a food source after every visit or increasing the number of landmarks around the food source, learning flights were significantly longer than before (Wei et al., 2002). Therefore, when aiming to control or manipulate learning flight durations, for example when comparing learning flights in two different contexts (e.g. at the nest and at a food source, see also Chapter 2), the design of landmark configurations and reward values needs to be carefully considered.

1.1.3 The role of compass information in view-based navigation

One effective way of dealing with ambiguities in the visual scene is to make use of compass information from the sky. Indeed, in order to efficiently match a memorised snapshot to its current view, the insect could benefit from knowing its current compass orientation and the one it had when it memorised the snapshot. For this reason, the acquisition of compass information by the insect is an important part of both the learning flights and return flights. It has been shown that honeybees learn a visual panorama in a celestial compass-based reference system and the two cues, panorama and compass, can substitute for each other (Towne and Moscrip, 2008). Bumblebees appear to have a strong preference for particular compass orientations while they face the nest both during learning and return flights (Hempel de Ibarra et al., 2009). Independent of the configurations of artificial landmarks, bumblebees preferentially faced northwards or southwards at different times of day. This diurnal change could help in optimising viewing conditions by enhancing the contrast of landmarks against brighter parts of the sky and/or simplifying view-matching during homing. This directional preference seems to be independent of the panorama, as it has been observed in different landscape settings, with variations in potential cues from the panorama that could be used by

bees, as well as in experiments where the panorama was hidden by high curtains placed around the table (Hempel de Ibarra et al., 2009).

Interestingly, these orientation preferences could also be influenced by the wind and it may be an indication that bumblebees use the nest odour as a cue in addition to visual information (Hempel de Ibarra et al., 2009). This hypothesis was also discussed by Philippides et al. (2013) to explain the differences in learning flight patterns of *Cerceris* and *Bombus.* The authors argued that these two ground nesting hymenopterans may rely on similar kinds of visual information during the orientation flights but that the odour of the solitary wasp's nest is less strong than that of the social bumblebee's nest, which precludes the wasp from using olfactory information as a navigation cue (Philippides et al., 2013). The use of this odour cue could, then, explain the presence of loops, which frequently take the bumblebee back to nest location during a learning flight, instead of arcs. Alternatively, the pattern of returning to the nest could also be an adaptation to the low vegetation in which bumblebees often locate their nest.

Previous work suggests that honeybees may use the magnetic field for compass guidance when the sky is clouded and the sun not visible, or indoors without celestial cues, to discriminate between panoramic patterns (Frier et al., 1996). Naïve bees did not choose the same preferred orientation when released under a tent and with an imposed magnetic field as compared to a test situation without the imposed field (Collett and Baron, 1994). Nevertheless, it seems that the magnetic field is only used during the first few flights. When magnets were removed after the initial learning flights, the bees kept the same orientation as during flights with the imposed magnetic field. It appears that when visual memories are fully formed, panoramic cues have a more important influence on the orientation than the conflicting compass cues.

The strongly-impacting compass cue used by bees is however the sky compass which uses the position of the sun as orientation guide. In many experiments it has been demonstrated that there is a causal relationship between the bees' waggle dance direction and the sun position derived either from viewing the sun directly or estimating its position from the distribution of polarised light in the sky (Dyer and Gould, 1981; Frisch, 1967). Do bumblebees use polarised light to maintain their North-South body orientation during their learning flights? And if they do use the polarised light, when during the learning flight do they refer to this sky compass? Do they need the compass information during the whole length of the learning flight or do they simply calibrate their orientation at the very beginning of the flight? These questions could unfortunately not be addressed in this thesis, as inside the greenhouse I could not detect a strong influence of compass information on body orientations.

1.1.4 Detection of the fixations in the tracked body orientations during learning flights

In the previous subsections, I have shown that orientation is an important factor of the learning flights and that during these flights, insects frequently turn back toward the location they just departed. Recent studies detected gaze fixations during the learning flights of both ground nesting wasps and bumblebees (Riabinina et al., 2014; Stürzl et al., 2016). For the studies decribed in this thesis, in order to conduct the analysis of learning flight trajectories, I automatized the detection of such fixations in the bumblebees' flights in order to detect any patterns in the bees' orientation during these fixations. Here, I explain the computational process and rationale I used to detect these fixations.

Gaze fixations can be detected in insects by tracking their body orientation, if the heads move very little, especially during flight, like in bumblebees (Riabinina et al., 2014). The bumblebee's head is largely aligned with the body most of the time. I was interested in gaze fixations because they may indicate instances when the insects learn views, particularly those relative to the goal, nest or flower. These views are likely to be learned for image matching during homing. Next, I explain the criteria for identifying specific segments of flights during which the retinal position of the goal remained nearly unchanged, which I term fixations. Gaze fixations in bumblebees have a minimum duration of 60 ms (Riabinina et al., 2014), therefore I considered here fixations that lasted at least 4 frames (for a 50 frames per second recording rate).

For each sequence of body orientations in a learning flight, I first took the angular position of the bee's longitudinal axis relative to the flower (φ) and scanned successive frames of each flight, noting the modular angular difference (diff φ) between adjacent frames, n and n+1. If diff φ was >3°, I repeated this calculation on the next pair of frames i.e. frames n+1 and n+2, continuing the process until diff φ was $\leq$3°. Such a small rotational difference indicated the

potential start of what I accept as a fixation. To test whether it was indeed a start, I added the next frame to the two-frame sample. If the modular difference between the minimum and maximum values of the sample of three frames (φ [diff.min.max]) was ≤3°, I added the next frame to the sample and again tested whether φ [diff.min.max] of the four-frame sample was ≤3°. This loop was repeated, sequentially adding frames until φ [diff.min.max] of the whole sample was >3°. Provided that the sample size of successive frames with φ [diff.min.max] ≤3° was ≥4 frames, the sample was included as a fixation and I recorded its duration and the median value of φ. I then continued to scan neighbouring frames until I encountered the start of another potential fixation (φ [diff]) ≤3°), when once more I tested whether these and subsequent frames met our criteria of a fixation. If they did not, the scanning of neighbouring frames was resumed from the 2nd frame after the potential start. This process continued until the end of the flight.

To choose an appropriate degree of variation I could allow within the fixation I compared different criteria ranging from 1° to 10° and for fixation durations from 1 to 10 frames in learning flights and return flights from trial 1 to 4 which are presented in Chapter 2. I then visually determined the best parameter evaluating (i) the histogram of the numbers of fixations depending on their durations (Figure 1.1 A, B), (ii) the histogram of the number inter-fixation moments depending on their length (Figure 1.1 C, D), (iii) the histogram of the number of flights depending on the proportion of their duration spent in fixations (Figure 1.1 E, F). Finally, I visually compared the graphs obtained with combinations of fixation parameters to eventually chose one of them. As example, the pair of parameters 5° and 4 frames presented a less sharp distribution of the length of the fixations (Figure 1.1 B) than the pair 3° and 4 frames (Figure 1.1 A), indicating that we detected longer fixations. However, with 5°, a larger proportion of fixations were separated from the next one by only 1 frame or less (Figure 1.1 C, D). Moreover, the proportions of the flights spent performing fixations

seemed unrealistically high using 5° (Figure 1.1 F) and more reasonable with a maximum variation of 3° (Figure 1.1 E). I, thus, decided to use the 3° and 4 frames criteria.

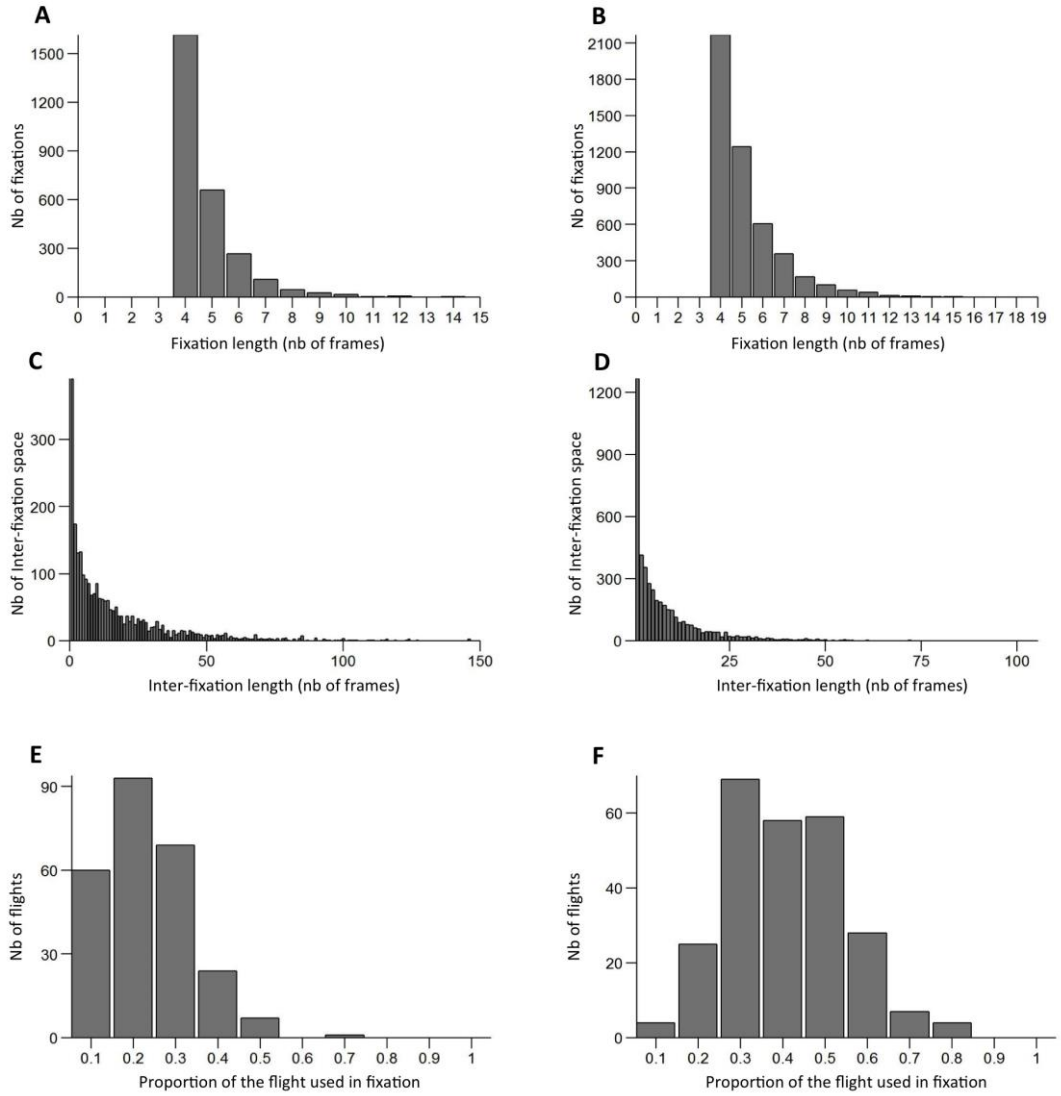


Figure 1.1. Distribution of the fixation lengths (A and B), distribution of inter-fixation lengths (C and D) and distribution of flights relative to their proportion used in performing fixations (E and F) using the parameters 3° maximum body orientation variation and 4 frames minimum fixation length (left column) and for 5° maximum body orientation variation and 4 frames minimum fixation length (right column). These graphs combine the learning and return flights 1 to 4 recorded during the experiment presented in Chapter 2.

1.2 Foraging behaviour of bumblebees

In the previous sections, I have presented evidence that bees and wasps perform an important number of their learning flights when departing a food source. Therefore, as one of the aim of this thesis is to investigate how bumblebees vary their learning flights when departing from flowers, we designed our protocol accordingly with the bumblebees' natural foraging behaviour.

1.2.1 Flower choice

Given that all the resources required for survival, development and reproduction are generally not gathered at the same location, animals have evolved a range of navigation mechanisms to effectively exploit resources in their environment. In social hymenopterans, only a small proportion of individuals in the colony have the responsibility to forage for the entire colony going out to find food that they bring back to the nest. Their exploration, navigation and foraging decision-making are determined by the location and distances travelled from the nest. Food sources can be scattered over large areas, nevertheless bees will not visit all the flowers available in a flower patch, even though multiple species of flower would offer good reward (Chittka et al., 1997; Heinrich, 1979). Aristotle already noticed that individual bees were more keen to forage on a single or a very limited number of flower species than to visit other species, a behaviour termed flower constancy. Later, Darwin (1876) proposed that knowledge of flower locations and of handling techniques for exploiting a flower could easily determine the bee's efficiency in foraging. Several field studies reported flower-constant behaviours in honeybees and in bumblebees (Bennett, 1883; Christy, 1883; Heinrich 1976; reviewed by Grant, 1950 and Waser, 1986), although the latter showed a less degree of specialisation and were seen more frequently visiting several flowers in a foraging bout. The analyses of the pollen brought back by both honeybees and bumblebees supported these

observations (Free, 1963; 1970), while experiments conducted in honeybees (Banschbach 1994, Hill, Wells, and Wells, 1997; Wells and Wells, 1986) and bumblebees with artificial flowers (Evans and Raine, 2014; Heinrich et al., 1977, Kunze and Gumbert, 2001) found that bees also show flower-constant behaviour in an artificial environment. In these experiments bees were trained to forage on flowers of a particular colour and were reluctant to switch to newly introduced flowers of different colours, even if these new flowers provided better rewards. Bumblebees appeared more prone than honeybees to switch from one flower type to another, as found in the field studies.

The evidence for selective pressures that led to the evolution of flower-constant behaviour is controversially discussed (Chittka et al., 1999). Whilst it is known that bees can learn several colours presented in succession or simultaneously as stimuli or in coloured patterns and recall learned associations over extended periods of time (Cheng and Wignall, 2006; Hempel de Ibarra et al., 2002; Menzel, 1968, 1969, 1979; Menzel et al., 1994, Nicholls et al., 2015), it has been proposed that flower constancy is limited by the memory capacity for storing and retrieving flowers handling techniques (Chittka et al., 1999). Indeed, some studies have shown that forcing bumblebees to forage on multiple types of flowers increased the bees' handling time of the flowers (Gegear and Laverty, 1995, 1998; Laverty, 1994; Woodward and Laverty, 1992). However, Gegear and Laverty (2001) argued that across all these studies, the bumblebees' handling time never returned to the level those of naïve bees and thus the insects retained at least part of the previously learnt handling techniques.

An alternative (although not mutually exclusive) explanation proposed is the search image hypothesis. Based on the observation that predator birds tended to eat sequences of the same cryptic moths, Tinbergen (1960) proposed that the predators needed to focus on the detection of very specific retinal stimuli to detect the presence of such cryptic preys. Later, several studies proposed that the same principle applied in the case of foraging bees looking for

rewarded flowers. Goulson (2000) added that flowers may appears cryptic to bees when other species of similar colour are present in the same flower patch.

However, this last explanation requires the bees to be already attracted toward specific flower visual characteristics. This attraction may arise from either innate colour preferences or association of these visual characteristics with the flower nectar rewards through past experience (Menzel, 1985; Giurfa et al., 1995; Raine and Chittka, 2007). Moreover, bees make foraging decisions based on the characteristic of the nectar rewards offered by flowers.

1.2.2 Reward value and flower choice

Dukas and Real (1993) used array of flowers filled with nectar or water to test the foraging decisions of bumblebees. They showed that bumblebees use the presence (nectar) or absence (water) of reward in a flower to decide whether to stay in the vicinity of this flower or to travel further to find the next flower to visit. Bees that experienced a rewarded flower were more prone to visit close flowers while bees that encountered a water filled flower had the tendency to look for flowers further away. Moreover, they observed that bumblebees travelled even greater distances if they experienced a sequence of flowers without reward. Waddington (1980) reported a similar behaviour in honeybees. But the difference between rewards does not need to be so extreme. Studies on several bee species have shown that bees fly further after visiting flowers with low nectar volume (Gegear and Thomson, 2004; Heinrich, 1979; Kadmon and Shmida, 1992). Low amounts of nectar in flowers do not only push the bumblebees to change location but also reduce their flower constancy. Using the bumblebees' flower handling time as measurement of the amount of nectar collected in wild flowers from a patch containing several species, Chittka and colleagues (1997) reported that bumblebees

were more prone to switch to a new flower species after visiting a flower that provided a low volume of nectar than after visiting a flower that contained a large amount of nectar. Honeybees and bumblebees also make foraging decisions based on nectar concentration. Cnaani and colleagues (2006) suggested that the effect of the nectar concentration may even be stronger than the effect of the nectar volume on bumblebees foraging decision. In a choice between two types of artificial flowers, bumblebees were more strongly attracted toward the flowers offering the highest reward if the rewards differed in concentration than if they differed in volume. Moreover, both honeybees and bumblebees appear to react to a decrease of the nectar concentration at a familiar feeder (Townsend-Mehler, Dyer, and Maida, 2011) by decreasing their visits to the feeder and increasing their tendency to find new feeders. Honeybees were more reluctant than bumblebees to switch feeders and needed a larger drop in nectar concentration.

Finally, the nectar concentration experienced by the bees seems to strongly influence their investment in learning the flower location, through the duration of their turn back and look behaviour (TBL). In 2002, Wei and colleagues studied how increasing the nectar concentration at a feeder affected the TBL behaviour of the honeybees. They first observed the decline in the duration of the bees' learning flights during the 10 to 15 first visits a feeder filled with a 0.5mol/L sucrose solution. All the bees were then relocated the bees 3 meters away where for one group of bees the sucrose concentration in the feeder was unchanged (control group) and for two other groups raised to 1 or to 2.25 mol/L. The three groups of bees increased their learning flight duration significantly and the flights of the 2.25 mol/L group was significantly longer than those of the control group. In a second experiment, three different groups of bees were trained to forage at feeders containing 0.5, 1 or 1.5 mol/L. Later, the three groups experienced a switch to the same sucrose concentration of 2.25mol/L as well as a relocation. This switch induced a significant increase of the learning flight durations of

all groups and the increase was significantly more for the group that first experienced 0.5mol/l. Thus, the increase in the length of the flights depends on the magnitude of the change in sucrose concentration. In a later experiment, Wei and Dyer (2009) confirmed that either an increase of nectar concentration or an in nectar volume could induce longer learning flights.

Based on the evidence presented above, it is known that bees are able to both estimate the quality of a flower and adapt their behaviour accordingly. Moreover, honeybees seem to increase their investment in learning a food source location after an increase in the quality of the reward offered. However, in the studies presented above, the food sources were slightly displaced after the switch in nectar quality. Thus, two questions arise: Do bees adapt their investment in their learning flights to the quality of the flower they departed for the first time? And do they re-elicit their learning flights after an increase of the nectar quality of a flower they already visited multiple times, even without a displacement of the flower location. These questions are addressed in Chapter 3.

1.2.3 Differences in life history traits of male and female bumblebees

Bumblebees belong to the genus *Bombus* of the subfamily *Apinae* and tribe *Bombini*, sister clade of the honeybees (*Apini*). Similar to most other bees, they collect nectar to supply the colony with sugar and the pollen needed to provide the larvae with a protein-rich diet (Goulson, 2010). Bumblebee colonies are a eusocial species with three castes – queen, workers and males. Colonies have an annual life cycle, and only young, mated queens survive from one year to next in order to found their own colonies in the following year. Unlike honeybees, temperate species of bumblebees only have colonies of few hundreds of individuals (typically 200-300).

The species on which we will focus here, *Bombus terrestris*, is native to the United Kingdom. Young queens and males emerge in colonies towards the end of the summer. After a successful mating, young queens hibernate underground during winter and look for a suitable nest sites in the spring. They usually use existent holes in the ground such as natural cavities or, more commonly, abandoned burrows of small mammals, which present the advantage of supplying material to build efficient thermal isolation with the residual mammal hairs (Goulson, 2010). The queen forages herself and feeds the first generation of workers. She compresses the pollen in a large ball in which she lays her first eggs (between 8 and 16) before to cover it with a layer of wax. She maintains the temperature of the ball over 30 degrees Celsius by keeping it in a close contact with her ventral surface and generating heat. The larvae hatch four days later and start consuming the ball of pollen while the queen will regularly forage to supply more pollen. After two weeks, the larvae build individual cocoons and start their pupal stage. During this time, the queen generally starts laying new batch of eggs. Two weeks later, the first workers emerge. The queen then stops her foraging activity while some of the workers pick up the task. The remaining part of the new work force helps the queen to take care of the broods.

The colony grows until the reaching its full size during summer. At the end of summer, the most successful nests produce both males and young queens. Because breeding future queens require an important amount of resources and a large number of workers, the less successful colonies often breed only males.

Differently, males are generally haploid. Some males can be diploid but homozygote for one specific loci (Duchateau et al., 1994), but they produce poor quality progeny (Duchateau and Mariën, 1995). The queen can actively control the fertilisation of her eggs and thus decide to produce either males or female individuals (Goulson, 2010). In addition, it has been proposed that the production of future queens depends on the production of a non-volatile unidentified

pheromone produced by the queen which, when present, will force the determination of the female larvae as worker (Alaux et al., 2006; Lopez-Vaamonde et al., 2007). As the female larvae need to be more fed frequently to become a future queen than to become a worker (Ribeiro et al., 1999), if the queen is removed or cannot be in contact with the brood, the female larvae will produce a pheromone stimulating the brood feeding behaviour (Pereboom et al., 2003). All this evidence suggests that the queen can to some degree control the production of the sexual individuals.

The time between the production of the first workers and the emergence of the first sexual individuals varies between colonies. Some colonies will breed sexual individuals only 10 days after the emergence of the first worker while other colonies switch to production of sexual individuals 14 days later (or 24 days after their first workers). The early colonies produce a large number of males and very few future queens while the late colonies seem to invest more in the production of queens (Duchateau and Velthuis, 1988).

Toward the end of a colony's life, after the production of sexual individuals has started, the workers will start laying their own eggs. Because they did not mate during their life, they cannot fertilize their eggs and all of them will develop into male individuals. This phenomenon, known as competition point, happens roughly 30 days after the emergence of the first workers in both early and late colonies (Duchateau and Velthuis, 1988). It is characterised with an ovarian development in the workers correlated with an increase of aggression behaviours against both the nest mates and the queen (Duchateau, 1989; Foster et al., 2004; Van Doorn, 1987), sometimes leading to matricide (Bourke, 1994).

Males will leave the nest and never come back (Paxton, 2005, Goulson, 2010). They migrate between 2 and 10km from their colony’s location (Kraus et al., 2009) presumably to avoid mating with their sisters as inbreeding appears to have extremely harmful effect on the fitness of the colonies (Gerloff and Schmid-Hempel, 2005; Whitehorn et al., 2009). Males, then

patrol regularly along routes, stopping near prominent landmarks near which they leave a pheromone (Goulson, 2010). This species-specific pheromone (Luxová et al., 2003) has the property of attracting the virgin queens, with a maximum effect when the male is 10 days old (Coppée et al., 2011). This scent-marking and patrolling strategy may also be combined with another one: the hilltoping, a strategy adopted by several other species in the Apidae family. Goulson et al. (2011) have noted that males were more frequent at the top than at the bottom of four hills in Scotland. They also propose that such behaviour would concentrate the population of males and help the virgin queens to rapidly find a male to mate before looking for a location suitable to their hibernation.

In the light of the bumblebees' specific life cycle, we can understand the importance for the workers to be highly efficient in their foraging activities in order to insure the rapid development of the colony and increase its chances to produce good sexual individuals to improve the colony's overall fitness. This explains the necessity for the individual to adapt their investment in their learning flights to the relevance of the location they are departing. Based on this idea, we designed the experiments presented in Chapter 2 and 3.

In addition, as the males do not participate to the colony's foraging effort but simply disperse to live alone and patrol regular routes for several days, one can see how it would be profitable for them to learn the location of good food sources along these routes. Therefore, we could expect them to perform a learning flight when leaving a quality flower. This hypothesis is explored in Chapter 4.

1.3 Thesis overview

Learning flights of hymenopteran insects are fascinating because they are a behaviour that is composed of a set of fixed responses and motor routines that ensure that each individual

learns about the spatial location of the goal. At the same time it allows sufficient flexibility so that this learning is adjusted to the unpredictable conditions of the habitat in which an insect might find itself. Given that the accurate description of these flights for a few species is available, including bumblebees, and we know that during these flights bees learn remarkably fast where the location of the nest or a feeding site is, it is possible to address more refined questions about when and how insects learn during learning flights and which aspects of learning flights differ between species, differences in the visual scene or behavioural context. Bumblebees are a particularly tractable species to study learning flights, as they can be obtained in large numbers and their colonies can be easily moved around or installed inside a standardised environment where it can be often easier to manipulate sensory stimuli. Individuals of both sexes can be marked and therefore tracked over extended time.

Here I aimed to understand how bumblebees *Bombus terrestris* vary their learning flights in two different behavioural contexts but in similar visual scenes and how these variations define the return to a learnt location. This is explored in depth in **Chapter 2**, where I compared the characteristics of the learning flights performed by the bumblebees when departing their nest with those of the learning flights they performed when leaving a flower. I also examined whether there are differences in the bees' returns at the two locations that can be due to these differences in the learning flights. In **Chapter 3**, the impact of the reward quality experienced at a feeding site on the learning and return flights was observed. In **Chapter 4**, the behaviour of male bumblebees departing their nest and a rewarded flower was studied for the first time showing that male bumblebees perform learning flights at flowers, one of the locations of importance to them.

Chapter 2

Comparing the learning flights of bumblebee workers (*Bombus terrestris*) leaving an artificial flower with those when leaving the nest

Abstract

Wasps and bees perform learning flights on leaving significant places like their nest and feeding sites during which they acquire information about the visual characteristics of these sites that can help guide the insects' returns. Because bumblebees, Bombus terrestris, nest in the ground and will collect nectar from low plants, it is possible to compare the learning flights evoked by the two goals in circumstances in which the visual surroundings of each goal are very similar. We have recorded the learning flights of individual bees as they learnt both goals set 5m apart. The locations of the inconspicuous nest hole and flower were each marked by the same set of landmarks, three black cylinders and a flat purple ring. Flights were recorded with two video cameras, one above each goal. The analysis reveals some structural similarities between flight manoeuvres at the two goals, but flights at the feeder are conspicuously shorter than those at the nest and the bees spend significantly less time close to the nest. In unrewarded tests bees were more persistent in locating the nest as compared to the flower, when the purple ring was removed, showing that they learned features on the ground during their departures from the nest. These differences may well be related to the permanence and uniqueness of a bee's nest compared with the more transient nature of flowers and their wider availability.

2.1 Introduction

Bees and wasps perform what are known as learning flights when they leave a significant location to which they will return. Such sites may be their nest (Collett, Hempel de Ibarra, Riabinina, & Philippides, 2013; Hempel de Ibarra, Philippides, Riabinina, & Collett, 2009; Philippides, Hempel de Ibarra, Riabinina, & Collett, 2013; Stürzl, Zeil, Boeddeker, & Hemmi, 2016; Zeil, 1993) or a feeding site (Lehrer, 1991, 1993; Collett & Lehrer, 1993), or, in the case of parasitic wasps, the location of a host's nest (Rosenheim, 1987). The learning flights are structured so that the insects learn enough about the surrounding landscape on a single flight to guide a successful return.

Because this basic function of the flight is similar across species and the goal to which the insect returns, there are many similarities between, for instance, the learning flight of the sand wasp *Cerceris* when leaving its nest (Zeil, 1993a) and *Vespula* when leaving a feeder (Collett and Lehrer, 1993). On the other hand, the nest and flowers have very different functions, properties and visual features. For instance, nest holes tend to be inconspicuous, but flowers often advertise themseleves to be conspicuous. Nests are mostly fixed and permanent, but flowers are more transient and there are often many of them. Learning flights to these different goals may be adapted to some of these differences.

Do hymenopterans adapt their learning flights to the nature of the location they departed from? The experiment described here is an attempt to look for possible adaptations in learning flights from the nest and a flower. To avoid being misled by species differences in learning flights, flights from the nest and the flower have been recorded using the same species, the bumblebee, *Bombus terrestris L.* This species is particularly appropriate as it both nests in the ground and will also forage at low lying flowers, like clover. That makes it possible to make the scene very similar at the nest and flower (see Material and Methods

section). These similar settings reduce the danger that differences between flights can be ascribed to differences in the scene (cf. Zeil 1993; Wei, Rafalko, & Dyer, 2002; Hempel,et al. 2009).

In this study, we examined bumblebees' learning flights from and return flights to the nest and an artificial flower during four successive foraging trips. The flights are compared both for differences in details of the manoeuvres at the two goals and in the ways that the flights change over successive visits. We also analysed tests designed to examine how well bees could locate the nest and flower.

Because of the intrinsic caharacteristics of the bumblebees' nest and the flowers, we hypothesised that the successive learning flights performed at these two types of locations would present some key differences. In addition, we added that these differences would affect the spatial accuracy of the bumblebees' memory.

Our predictions were that bumblebees perform shorter and less elaborate learning flights when departing a flower than when departing their nest because of the very conspicuous nature of the flowers opposed to the inconspicuous nature of their nest. We also predicted that because of these shorter flights, the bumblebees have less accurate memories of the locations of the flowers they visited than of their nest.

However, because the learning flights perfomed at the two types of locations serve the same purpose, we also expected them to share some commun characteristics.

2.2 Material and Methods

2.2.1 Preliminary experiments and protocol development

The experiments we discuss in this sub-chapter were designed to compare learning flights at the nest and feeder. However, this work evinced important methodological limitations, which were addressed to improve the methods of the main experiments presented in sub-chapter 2.2.2. Two experiments were conducted and analysed. In the first experiment, we asked whether learning flights differ when bees departed the nest and the flower. The surroundings of the nest and flower were kept identical. Both tables had the same white cover, and the locations of nest and flower were rather inconspicuous and only marked by three black cylinders around the nest or flower. In the second experiment, we removed the three cylinders from the nest location and replaced them with a black-white patterned ring around the nest hole. The flower was inconspicuous, as before, and surrounded by three black cylinders. We wanted to know whether learning flights would differ when the views at the nest and flower location differed.

Experiments were conducted in the greenhouse in November-December 2013 and in May 2014, in a 8 x 24 m space (twice the size of the one used during the main experiment presented in sub-chapter 2.2.2). During the experimental periods, the temperature in the greenhouse varied between 13 and 40 degrees. Two tables (appx. 1.5x1.5m) were placed six meters apart (Figure 2.1) and covered by identical white mats, that were of a similar make as those used by Hempel de Ibarra et al. (2009). To simulate ground-nesting conditions, the colony was placed under the nest table and connected to the hole in the middle of the table via a tube. In the centre of the second table, we placed an inconspicuous flower that was made from pipette tip plugged into a thin plastic tube that was connected to a syringe dispensing 50% sucrose solution.

In both experiments, the procedure had four steps that were repeated in each of the four trials. Firstly, the individually marked bee was released from the nest, performed a learning flight and flew off into the greenhouse. After a brief period of flying it was then caught and very gently placed on the inconspicuous flower until it started drinking. After it finished drinking, the bee

departed the flower performing a learning flight and flying off. The bee was caught and placed back into the colony.

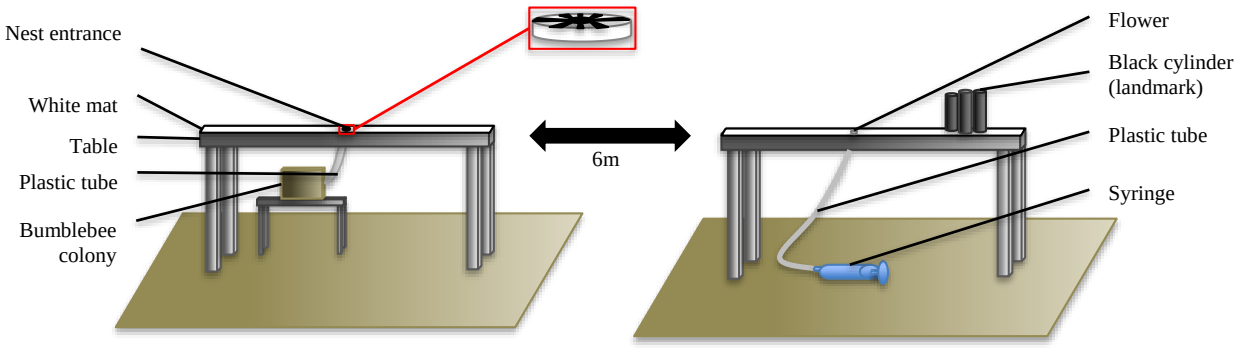


Figure 2.1. Schematic representation of our experimental setup during the second experiment. The nest hole was surrounded by a black-white patterned ring, whilst the inconspicuous flower was surrounded by three cylinders.

In the first experiment both the nest hole and the flower were surrounded by identical sets of three black cylinders (5cm diameter, 17cm high) placed at a distance of 22 centimetres each and separated by 60 degrees. The central cylinder was positioned approximately North from the nest and flower (aligned with the walls of the greenhouse, see also Figure 2.3 and 2.4). In the second experiment, the nest hole was marked with a flat ring displaying a black-white radial pattern, whilst the flower location was kept as in the first experiment – invisible and surrounded by three black cylinders (Figure 2.1).

We found that the first learning flight at the flower was much shorter (median 2.14s, IQR=2.34) than at the nest (median 11.30s, IQR=2.63) (Wilcoxon test, N=7, W=132, $p<0.001$) (Figure 2.2). As expected, the flights on subsequent trials were shorter. This finding was replicated in the second experiment. The first learning flights at the flower were shorter (median 2.28s, IQR=3.76) than the first learning flights at the nest (median 13.46s, IQR =3.85), even though the views differed between the two locations and learning at the first location could have facilitated learning at the second location (Wilcoxon test, N=6, W=49,

p<0.001). This indicates that the flower learning flights were not shorter simply because of the similarity between the views at the nest and flower in the first experiment.

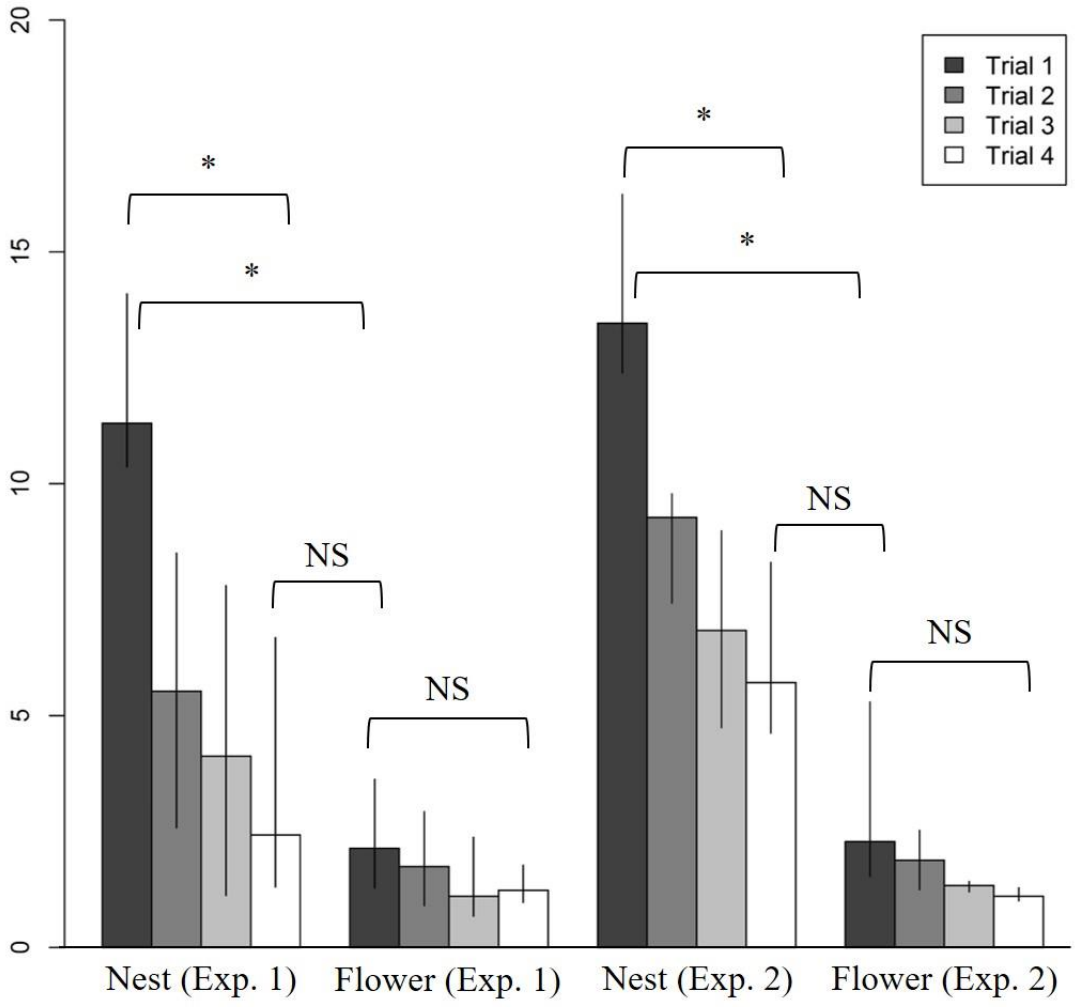


Figure 2.2. Median durations of learning flights when departing the nest or inconspicuous flower. In Experiment 1 the nest and flower were surrounded by three cylinders. In Experiment 2 only the flower was surrounded by the same three cylinders, whilst the nest hole was marked by a ring with a black-white radial pattern.

However, the experiments revealed some important limitations. The greenhouse was too large and we lost too many bees that hid or were predated by spiders in the far corners and ridges, or found tiny holes to squeeze through and escape. This problem was fixed for the main experiments by splitting off a smaller compartment with netting, which allowed us to

detect and catch many more bees that stopped for a rest. Netting off the ceiling reduced the number of escaping or predated bees.

The white mats had to be replaced and cleaned frequently. It was also difficult to control for potential foot prints around the flower that could have influenced the bees' behaviour. Therefore, we changed the cover to white gravel which could be raked regularly in order to prevent the accumulation of chemical cues at any of the two locations.

We initially tried to let the bee find the flower by themselves after the first trial. However, they never approached the flower table, which seems to be caused by the lack of a visual display. Therefore, we had to recapture the bees and place them on the feeder for every trial. For the final experiment we decided to use a purple ring as an artificial flower display in order to enable the bees to return to the flower by themselves and to place a similar ring around the nest to maintain the visual similarity between the two locations.

2.2.2 Main experiments

The main experiments were conducted between June and August 2015 in a greenhouse (8x12m floor area) on the Streatham Campus, University of Exeter (Figure 2.3). The temperature inside the greenhouse varied between 18 and 35 degrees. The presence of tall tree edges on the East and South sides and the fact that the main building was on the North side of the greenhouse (see Figure 2.3) lead to a more important luminosity coming from the West than from any other side of the greenhouse.

We experimented with worker bees from 4 colonies of *Bombus terrestris audax* supplied by Koppert UK. To identify individual workers participating in the experiments, each bee was marked with a numbered colour tag.

Bombus terrestris is a ground-nesting species that leaves its nest through a hole in the ground. To mimic this situation, we placed a colony in its nest box under a 'nest' table and recorded the flights of workers when they left their nest through a hole in the centre of a table and later returned to the nest hole. We also recorded the bees' departures after they had fed from a flat artificial flower on top of another, 'flower', table and their approaches to the flower on subsequent visits. The nest and flower tables were about 5 m apart.

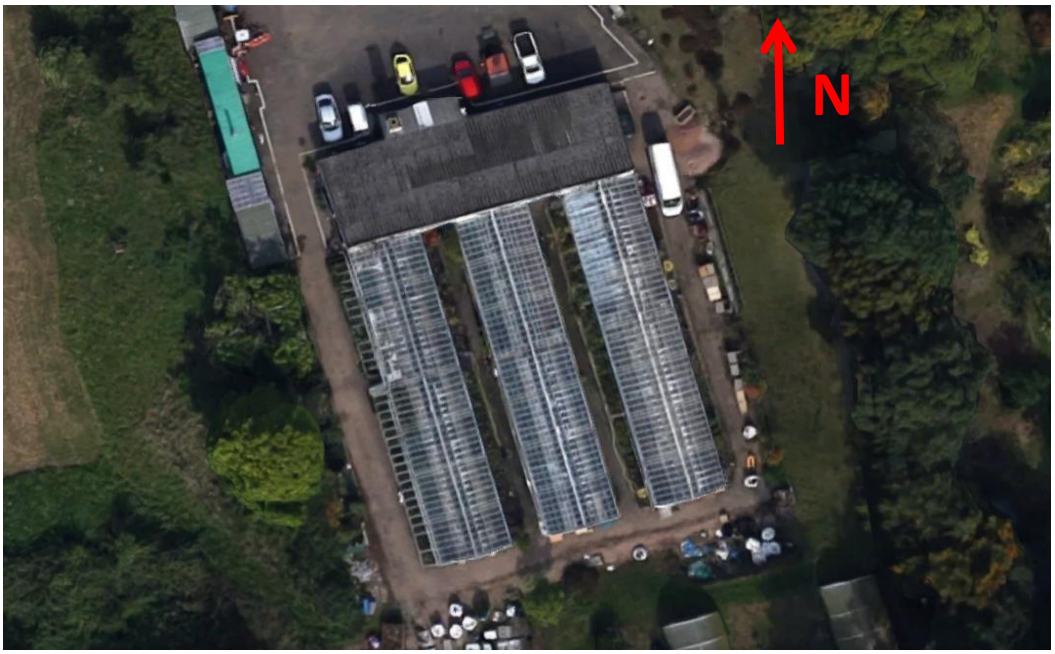


Figure 2.3. Satellite view of the greenhouse where the experiments were conducted. The red arrow indicates the real North direction. The experimental tables were set at the southern end of the West greenhouse. (©Goggle Earth)

We took pains to make the two tables visually very similar. To enhance the visibility of the bees when recorded and to provide visual texture to stabilise the bees' flight, both table tops were covered with white gravel. On top of each table, we placed a purple plastic ring (5cm diameter) which on the nest table encircled the nest entrance and on the flower table formed the artificial flower with an Eppendorf tube filled with 50% (w/w) sucrose solution in the

centre of the ring. Three black cylinders (5x17cm) placed at 22cm from the centre of the ring were distributed in a 60 degrees arc around it with the central cylinder roughly north of the purple ring (Figure 2.4). The start of the training procedure involved a 'training table' which in some respects looked different from the flower table. It had the same artificial flower in the centre, but no cylinders. And instead of using white gravel to provide visual texture to stabilise the bee's flight, the visual texture came from a tufted white bathmat spread over the table top.

The bees' flight above each table as they left or approached the nest or flower was recorded with video cameras (Panasonic HC-V720, 50 fps) that were hung under the ceiling (1.35 m above the surface of the tables). An area of about 70x90cm was captured in an image of 1920 pixels by 1080 pixels.

Figure 2.4. Picture of the experimental tables set up in the greenhouse. In the foreground is the nest table and in the background is the flower table. The black cylinders are visible on each table as well as the purple plastic rings marking the position of each location.

2.2.2.1 Training

The workers on leaving the nest entrance performed a learning flight. After the flight they usually landed inside the greenhouse on the roof where they were caught. After capture on their first departure, the marked bees were placed on the flower on the training table and allowed to drink ad libitum before leaving. They generally performed a learning flight on leaving the flower during which they had the opportunity to memorise the colour and shape of the rewarded flower. The bees then returned to the nest table and entered the nest hole. In most of the experiments the training table was then hidden under a black cover.
After their initial training, bees left the nest for a second foraging flight and flew around the greenhouse with the training table hidden from view. 19 bees found and fed from the flower on the flower table. After feeding, the bees left, normally performing a learning flight. They usually flew around for some time before returning to the nest. To prevent further learning about the flower on that foraging trip, they were prevented from revisiting the flower. After four more foraging trips, in which the bees' departures and approaches from and to the nest and flower were recorded, the bees were tested.

2.2.2.2 Tests

The first test (Test: nest no ring) was conducted on the bees' return to the nest at the end of the seventh foraging trip. The purple ring was removed and the nest entrance closed with a plastic board that was covered with gravel (Figure 2.5). The array of three cylinders was shifted without rotation to a new position. The bees' flight as it searched for the nest hole was recorded until the bees lost motivation to approach the location, the experimenters visually estimated that the bees were repeating the same search or another bee attempted to return to

the location. At the end of the test, the nest entrance was opened, the ring and cylinders were replaced around the nest hole and the bee allowed to enter the nest. On their eighth foraging trip, the same test was conducted at the flower table (Test flower no ring). After the test, the normal arrangement was restored and the bee was allowed to feed from the flower and to return to the nest. On the bee's ninth foraging trip, another test (Test: nest with ring) was

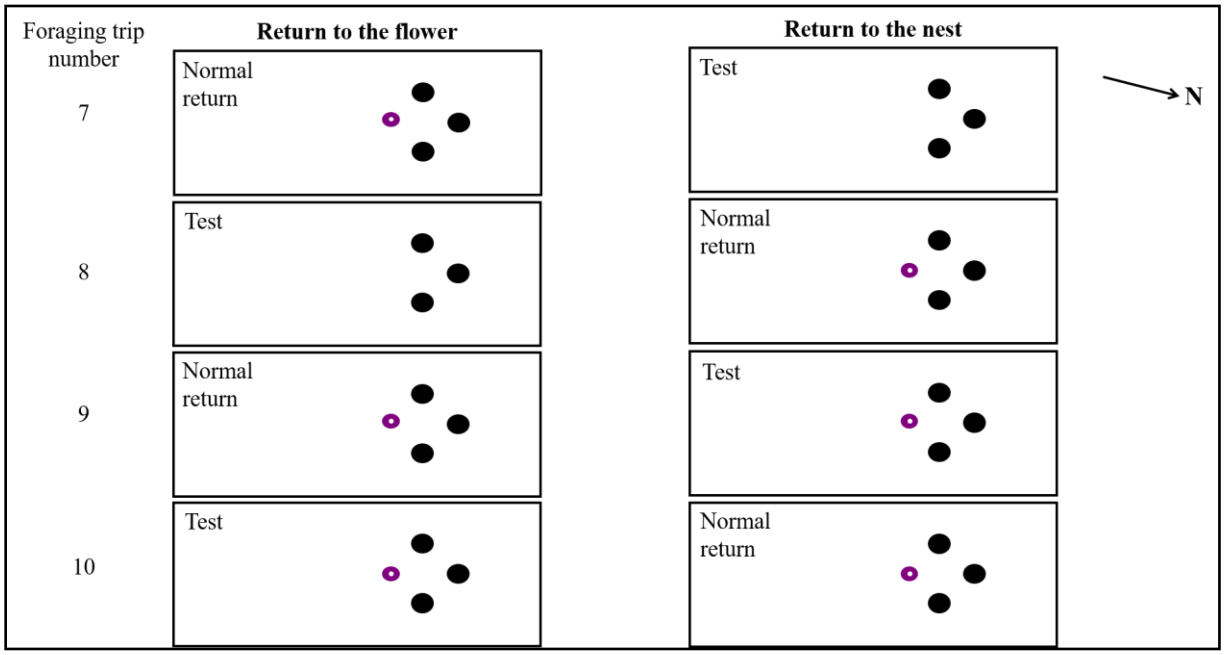


Figure 2.5. Schematic of the last foraging trips showing the order of the test without and with the purple plastic ring placed on the experimental tables. The rectangles represent top view of the flower table (left column) and nest table (right column). The black circles represent the three cylinders. The purple circles indicate that the purple ring was on the table during the bees' approaches. The nature of the return is indicated at the top right corner of the tables. During the "Normal returns", the bees could access their nest or feed on the flower. During the "Tests", the nest entrance was hidden and the flower was unrewarded. The black arrow indicates the direction of the real North.

conducted at the nest. The array of cylinders was again shifted in the same manner as before except that another clean ring was present and placed correctly relative to the cylinders. On the bees' tenth foraging trip the analogous test was conducted at the flower (Test: flower with ring).

2.2.2.3 Data analysis

The positions and orientations of the bees were extracted using custom-written codes in Matlab (Philippides et al. 2013). This code gave us the bee's position coordinates for each frames and its orientation. We were then able to compute the trajectory length, duration of the flight as well as the speed of the bee.

We were also able to detect the bees' fixations for at least 80ms of a particular direction (+/- 3 deg) relative to the nest, the flower, the cylinders or any other point of the table (virtual cylinders for example). The full computational process for this purpose is described in the Chapter 1.

For the tests, the video analyses were performed using Adobe Premiere to measure times and bouts duration and Photoshop CS6 to collect the coordinates of the bees' landings. We separated the landings in 3 different categories. 1) the landings following an approach of the goal. These were characterised by the fact that the bees were coming from the edges of the table and flew toward the goal before to land. 2) the repeated landings which were landings shortly following a landing of type 1) and generally performed in the vicinity of the first landings as the bees are searching a particular area. Finally, 3) the landings at the feet of the cylinders. These landings were of little interest to indicate the accuracy of the bees' searches as it seems unlikely that the bees were confused to the point of searching the goal in a place where the panorama was radically different from the one at the relative goal location. Thus,

this last type of landings was excluded from our analyses. Because the bees did not spend an equal time on the two experimental tables, the numbers of landings had to be divided by total sum of the searching bouts on each table.

Because the landings may not be the only indication of the location where the bees were searching for the nest or the flower, we also looked at the locations where the bees were slowing down during their searches. For each test of each bee, we visually chose the first bout during which the insect slowed down to search the nest or the flower. We extracted the bee's trajectory of this videos until the bee either left the camera view or landed on the table. If the bee did not land in this bout, we ended the clip when the insect left the camera view. We then proceeded to detect the particular location and orientation of the "slow down points" of this trajectory presenting the lowest ground speed. We first computed the Euclidian distance between the bee's position on each frame and its position in the previous one and the speed by dividing the distance by 0.02s (as the video was recorded at 50 fps). Because the resulting speed profile was very noisy, we applied a LOESS smoothing technique based on locally weighted regressions on the speed data using the "loess" function in R (3.3.0). Using the R function "diff", we subtracted each value of the smoothed data to the next one to obtain positive difference values when the bee increased its speed and negative difference values when the speed decreased. The "sign" function then converted the positive values into 1 and the negative values into -1. Running a loop though the succession of frames checking when a -1 value was followed by a 1, we could identify the frames on which the bees were changing their behaviour from deceleration to acceleration. Among these particular frames, we selected up to three instances per trajectory where a bee presented the lowest smoothed speed (or "slow-down-points") and determined the corresponding spatial coordinate and body orientation.

We used R (3.3.0) to analyse the data. Due to the non-parametric nature of our data, we applied GEE models using the "geepack" package. We also used the package "Circular" to compute circular statistics such as circular means and the mean resultant length (rho). We also used Wilcoxon tests to perform the planned contrast comparison.

For the training data, we only kept for the analyses the flights during which the bees did not have trouble to fly. Problems to fly were very characteristics and easily identifiable. Each video was checked visually and we excluded the flights presenting a bee that failed to gain altitude, struggled to manoeuvre and/or crash landed during their departure. Moreover, to be able to apply paired tests for the post hoc analyses, we frequently needed to exclude flights that did not have a matching flight to pair with.

When analysing the bees' returns to each locations, we kept only the approaches that followed a satisfying learning flights during the previous departure at the location. Four more flights were not included due to a camera problem during the experiment that lead to an interruption of the recording before the return of the bees. Finally, for the post hoc paired tests, we excluded the unpaired flights.

In the following sub-chapters, we will refer to the compass orientations relative to the array of cylinders, with the axis between the nest or the flower and central cylinder determining the 0 degree reference. When describing the bees' orientations relative to a particular cylinder, we will designate them by their position relative to the central cylinder as seen from the nest or the flower (i.e. left, central and right cylinder).

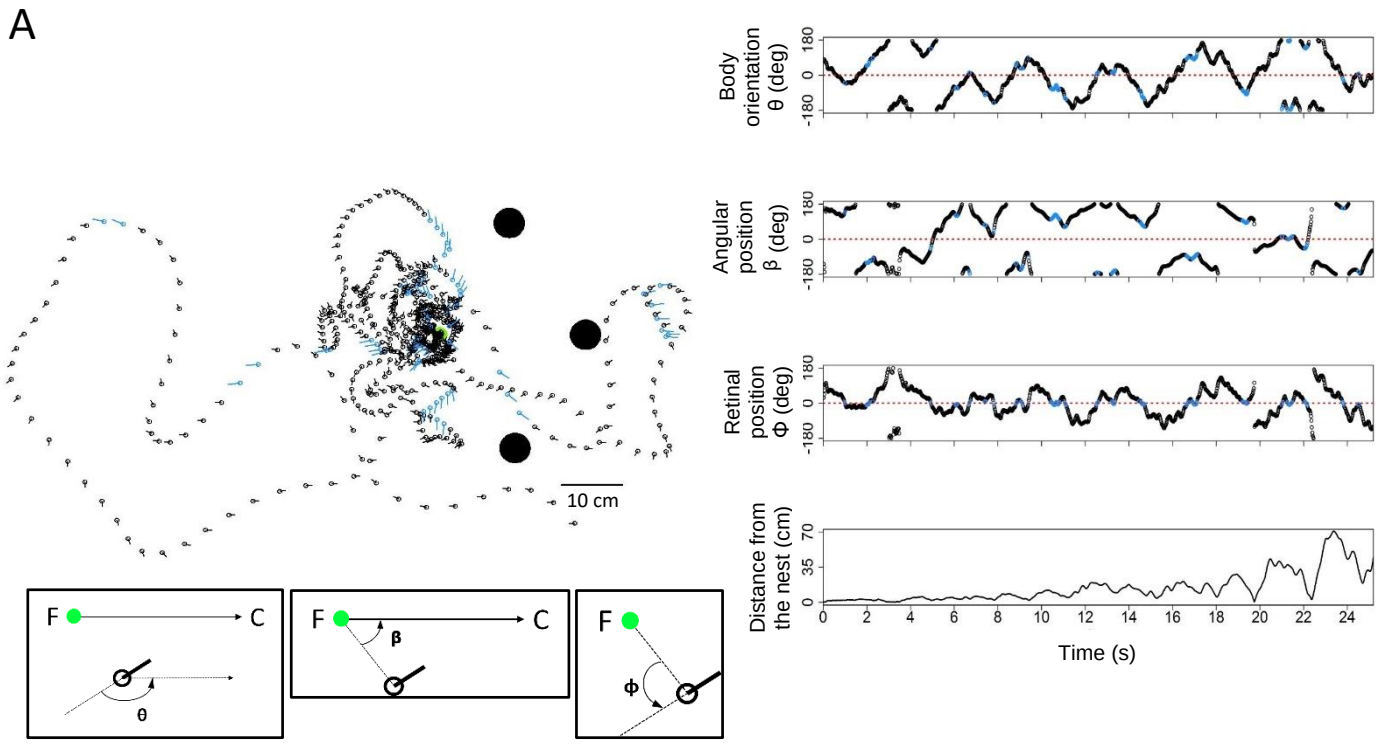
2.3 Results

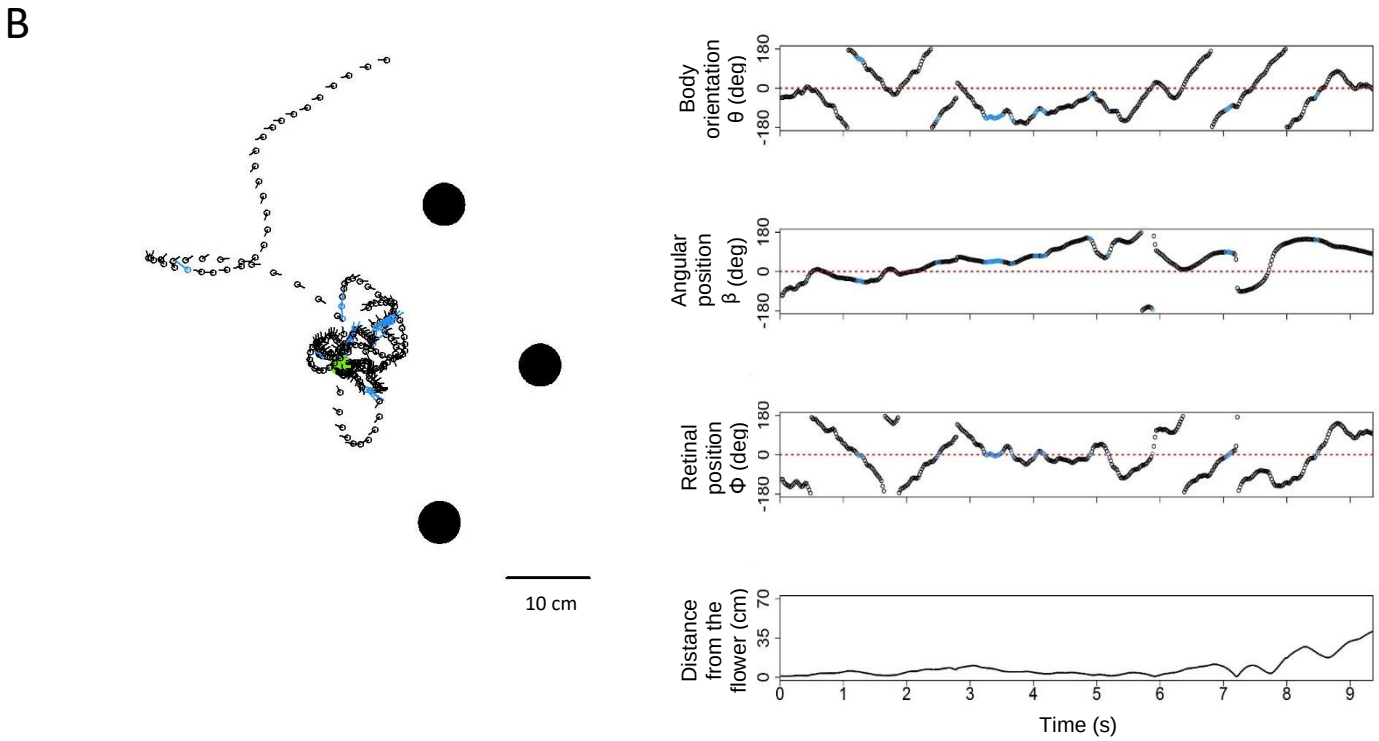
Learning flights on leaving the nest have been described before (Hempel de Ibarra *et al.*, 2009; Philippides *et al.*, 2013; Collett *et al.*, 2013; Riabinina *et al*., 2014). My aim is to compare these flights with those performed when the bees leave a flower for the first few times. To do so I have examined some features of learning flights that have already been explored and also compared characteristics of the flights that have not been analysed before.

2.3.1 General similarities and obvious differences between flights from nest and flower

The example flights of one bee (Figure 2.6) illustrate some general features of the bees' learning flights. During its first learning flight from the nest (Figure 2.6A) and from the flower (Figure 2.6B), the bee gradually flew away from these locations. For a significant part of these flights, the bee remained close to the nest or flower. In this first phase, it stayed within about 5 cm of the departure point for the first 30 to 40% of the flight (Figure 2.6A, B distance plot. See also Figure 2.7D). In the second phase of the flight, the bee gradually gained distance from the nest or flower, periodically returning all or partway to the departure point in a series of loops. At the flower, the bee performed fewer loops than at the nest. The loops were smaller and mostly occurred close to the flower after which the bee flew directly out of the video frame, whereas at the nest loops of increasing sizes took the bee beyond the camera's view. During segments of the 2nd phase of the flights, as the bee returned to the nest or the flower, it frequently faced (±20^{o}) the departure point. Across both phases of its first flights, the bee faced the nest 23 times and the flower 11 times (see the number of crossing of the 0 line in the plot of ϕ). As first described in wasps (Zeil, 1993a), the bee tended to rotate its body orientation (θ) at a similar rate throughout the flight, now and then reversing its

direction of rotation. This feature occurred in both flights from the feeder and the nest. When flying in arcs around the nest, wasps modulate the angle of their body orientation relative to the nest in a distinctive pattern (Zeil, 1993a). Because bumblebee loops are not always centred on the nest, the pattern, as in the example bee, is often not obvious (see plot of β). The pattern is even less clear on the bee's departure from the flower. During the sequence of foraging trips, the flights became shorter. The fourth learning flight at the nest resembled the first flight in its frequent nest facing (Figure 2.6C, 18 times) and because the flight was shorter with fewer loops it was also similar to the bee's first flight from the feeder, apart from the more frequent nest facing (18 vs.11 times). The fourth flight from the flower was almost a straight departure with no looking back except for a few frames at the start of the flight (Figure 2.6D, plot of ϕ).


A
10 cm
F
C
θ
β
ϕ
Body orientation θ (deg)
Angular position β (deg)
Retinal position Φ (deg)
Distance from the nest (cm)
Time (s)


B
10 cm
Body orientation θ (deg)
Angular position β (deg)
Retinal position Φ (deg)
Distance from the flower (cm)
Time (s)

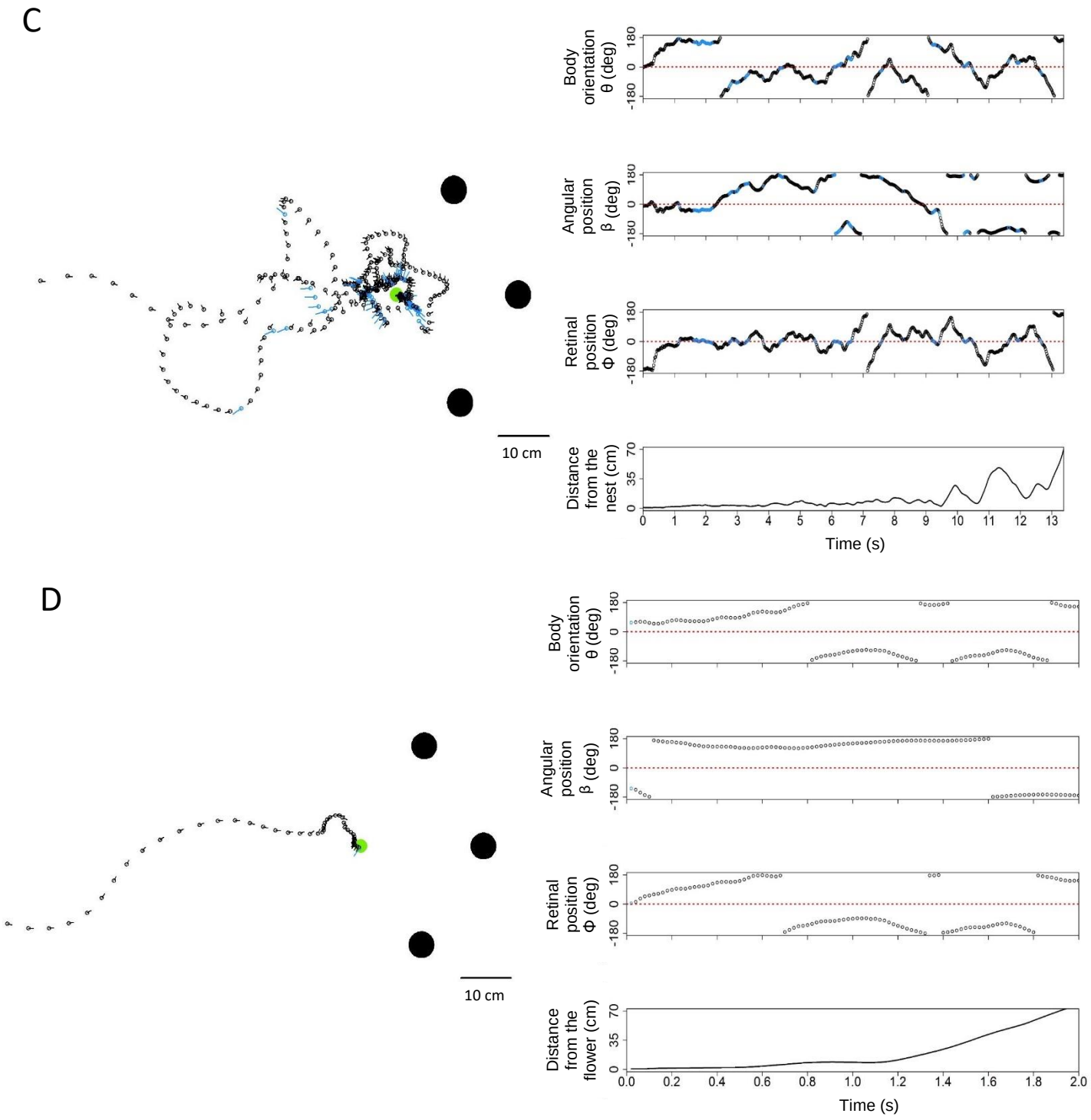


Figure 2.6. On the left: Top view of the trajectory of the bee FB4 during her first departure from the nest (A) and the flower (B), and the fourth departure from the nest (C) and the flower (D). The large black filled circles represent the position of the cylinders. The green circle shows the location of the nest or the flower. The empty circles are the positions of the bee plotted every two frames and the tails represent the orientation of the bee. The blue circles represent the frames on which the bee is oriented toward the nest or the flower (± 10°). On the right: Bees' cardinal orientation, orientation relative to nest or flower, retinal position of the nest or flower and distance from the nest or flower during the learning flights. Insets: Illustrations of the angles corresponding to the body orientation relative to the line between the flower and central landmark (F-C) (θ), angular position (β) and the position of the nest or the flower relative to the bee's longitudinal axis (ϕ, 'Retinal' position for short). Green circles indicate the nest or the flower. The arrows points in the positive direction.

2.3.2 How do simple parameters of the learning flights differ between the nest and the flower location?

As the example flights suggest, several key parameters of the bees' flights vary according to whether bees are leaving the nest or the flower. First, the bees performed longer lasting flights at the nest than at the flower (table 2.1.1) (Figure 2.7A). The median duration of the first flights at the nest was 12.44s, IQR=5.10 and the median duration of the first flight at the flower was 6.26s, IQR=4.05 (Wilcoxon, N=15, W=4, z=-3.18, p=0.001). Similarly, the fourth learning flights at the nest (median 3.04s, IQR=2.95) were significantly longer than the fourth learning flights at the flower (median 1.20, IQR=0.0.68; Wilcoxon, N=13, W=3, z=-2.97, p=0.003). Both flower and nest flights became significantly shorter over successive visits (1st vs 4th flight at nest: Wilcoxon, N=12, W=80, z=-3.06, p=0.002; at flower: N=18, W=200, z=-3.29, p=0.001).

Table 2.1. GEE modelling examining the influence of various predictors on several learning flights parameters. Entry 1: The flight duration decrease over trials while the learning flights at the nest were longer than the learning flights at the flower. Entry 2 and 3: The bees' mean ground speed when crossing radiuses of increasing distances from the goal. Bees' speed increased with their distance from the nest and the flower (entry 2). The bees were globally slower during their first flights (entry 2) but their speed did not significantly vary during the subsequent trials (entry 3). Entry 4: the trajectory length of the learning flights increased over trial and were longer at the nest than at the flower location. Entry 5: Bees' median distances from the nest or the flower within each tenth of the learning flights. The bees increased their distance from the two goals during their learning flights.

	Dependent Variables	Predictors	Estimates	Standard Error	$\chi 2$	*df*	*P*
1	Flight duration	Trial	-2.209	0.354	82.4	1	**<0.001**
		Goal Nest	6.217	0.685	39	1	**<0.001**
2	Ground speed	Radius	3.07	3.37	304.71	1	**<0.001**
		Goal Nest	3.1	3.93	0.62	1	0.43
		Trial 2	11.39	3.54	10.36	1	**0.001**
		Trial 3	15.62	4.42	12.5	1	**<0.001**
		Trial 4	15.37	3.3	21.66	1	**<0.001**
3	Ground speed	Radius	3.07	3.37	304.71	1	**<0.001**
		Goal Nest	3.1	3.93	0.62	1	0.43
		Trial 1	-11.38	3.54	10.36	1	**0.001**
		Trial 3	4.24	3.32	1.63	1	0.2
		Trial 4	3.98	3.24	1.51	1	0.22
4	Trajectory length	Trial	-35.4	5.2	46.3	1	**<0.001**
		Goal Nest	116.4	10.1	134	1	**<0.001**
5	Median distance	Flight section	27.752	1.247	496	1	**<0.001**

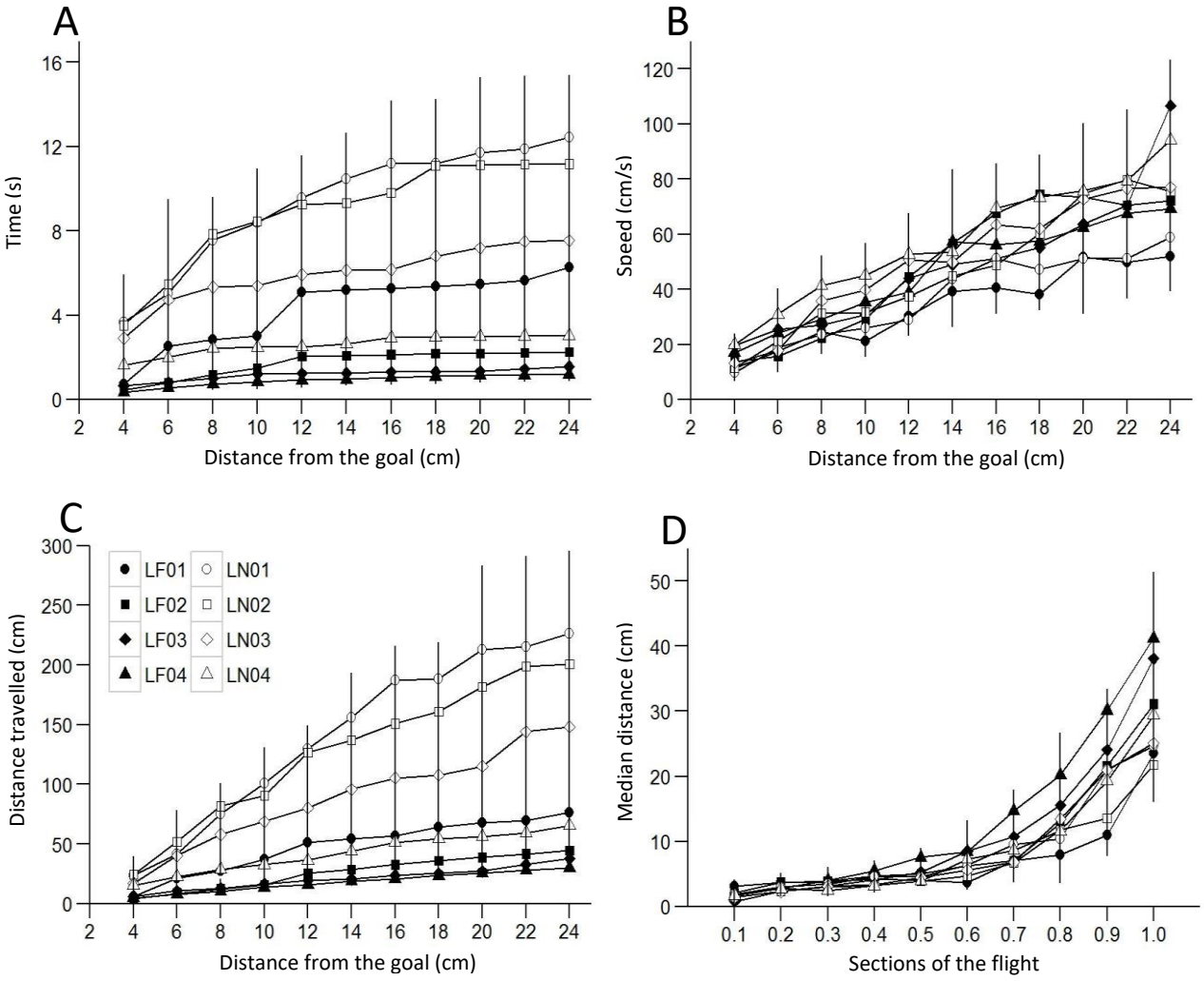


Figure 2.7. A: Median times taken by the bees before to cross different distances from the nest or the flower during the learning flights 1 to 4 at the nest (white symbols) and at the flower location (black symbols). The times are measured before the bees crossed a circle of a given radius to a maximum of 24 cm. B: Medians of each bee's mean speed when crossing different distances from the nest or the flower for the first time ± 2 frames. C: Median trajectory length flown by the bees before to cross different distances from the nest or the flower. D: Medians of the bees' median distances from the goal within each tenth of the learning flights. For each individual, the flight was divided in ten equal parts in duration and the bee's median distance from the goal was computed for each of these ten sections. The legend for the four graphs is shown in figure C. Here, and later in the thesis, the learning flights at the flower 1 to 4 are called LF01, LF02, LF03 and LF04. The learning flights at the nest 1 to 4 are called LN01, LN02, LN03 and LN04.

Exactly the same pattern was seen in the lengths of the trajectories (Figure 2.7C) (table 2.1.4). The median length of the first learning flights at the nest was 226.31cm, IQR=107.19 and at the flower it was 76.54cm, IQR=40.14 (Wilcoxon, N=14, W=2, z=-3.17, p=0.002). The length of the fourth trajectories at the nest (median 65.49cm, IQR=109.81) were also longer than the fourth ones at the flower (median 29.96cm, IQR=3.23) (Wilcoxon, N=13, W=0, z=-3.18, p=0.001). Again lengths reduced over successive visits (1^{st} vs. 4^{th} flights, nest: Wilcoxon, N=18, W=200, z=-3.38, p=7e-04; flower: Wilcoxon, N=11, W=70, z=-2.93, p=0.003).

The bees flew at similar speeds when leaving the nest and flower (Figure 2.7B). But a GEE model showed that within each trial speed increased with the bees' distance from the nest (table 2.1.2). Across trials speeds also varied: bees were significantly slower during the first trial than during the following trials, but thereafter speeds were similar (table 2.1.3).

The division of the flights into two phases seen in the example plot, the first with the bee remaining close to the nest or flower and the second phase, in which distance from the departure point increases, is more obvious when flight durations are normalised and data from many flights are superimposed (Figure 2.7D). A visual inspection of the figure 2.7D informed us that for about the first third of the flight the bee stays within about 5 cm of the departure point (apart from the fourth flight from the flower) and only after that does the bee gradually fly further away. For this reason, in much of the following analysis, the two phases are considered separately.

In both phases of learning flights from the nest, bees tend as a group to fly in all compass directions as indicated by the flat distribution of directions in Figure 2.8 and 2.9. The pattern differed in departures from the flower. The distribution of directions was uniform (as shown by the absence of clear peak in the distribution) in the first learning flight but changed progressively over later flights generating a growing peak to the opposite direction of the

cylinder array, particularly in the second phase. This peak is likely to correspond to the bees' departure direction from the flower table, suggesting that in these later learning flights visual information is no longer acquired in the second phase.

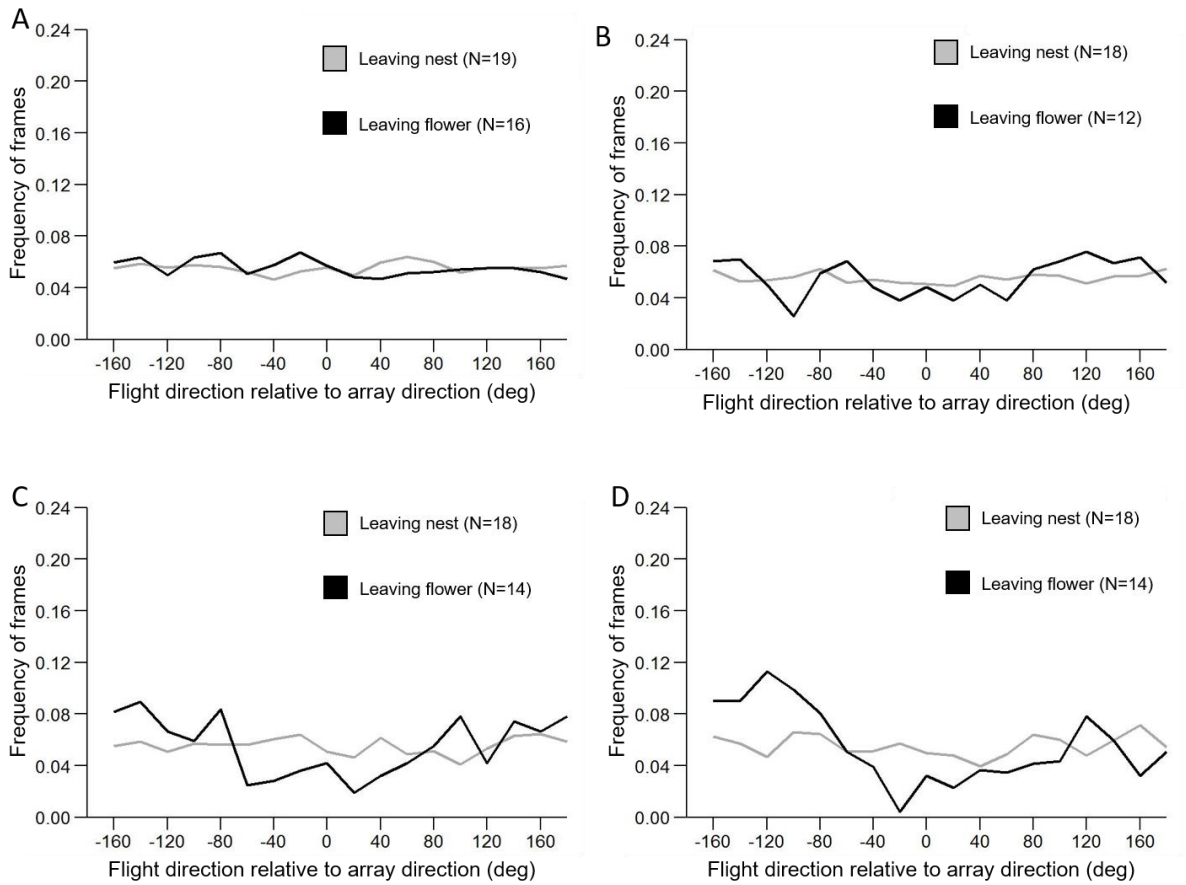


Figure 2.8. Frequency distribution of the bees' flight directions relative to array-direction for the early part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°.

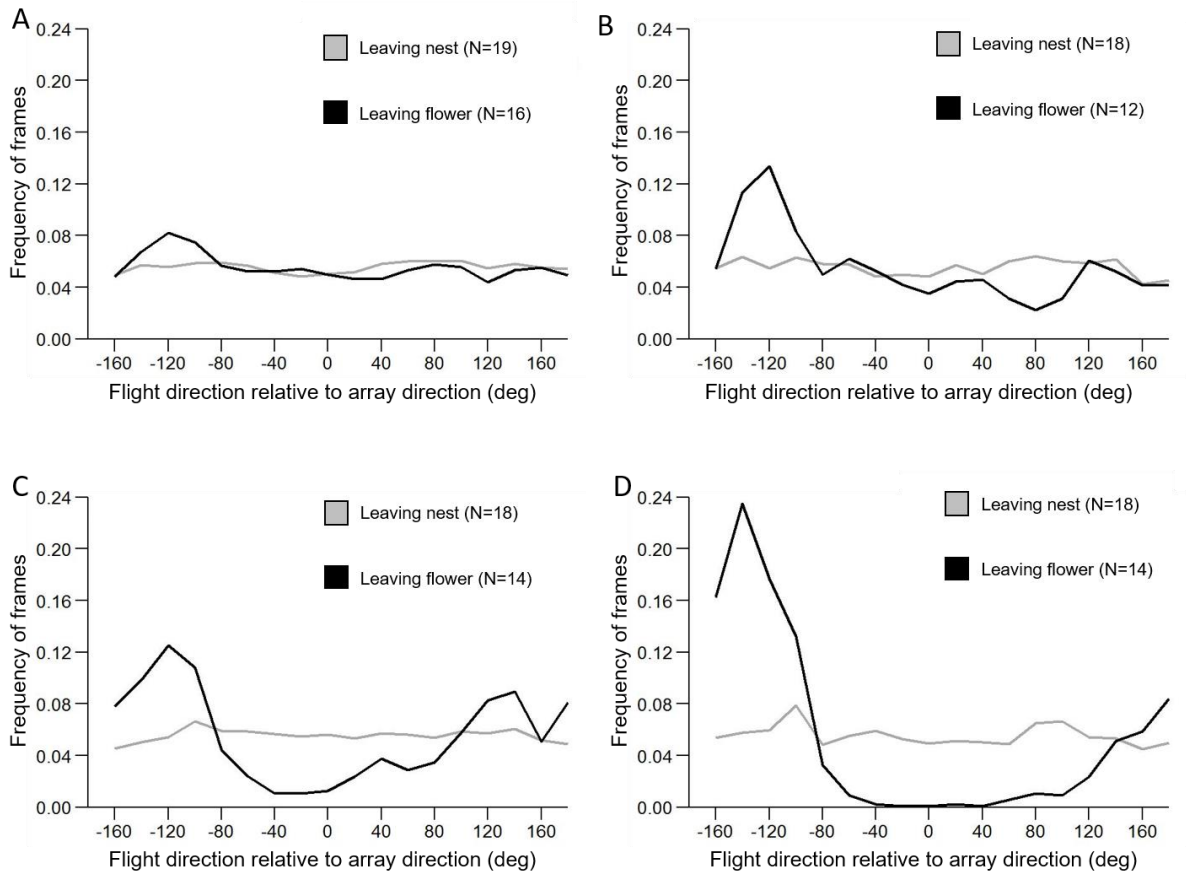


Figure 2.9. Frequency distribution of the bees' flight directions relative to array-direction for the late part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°.

The bees' flight direction relative to the goal when leaving the nest also differs between the first and second phases of the learning flight. A visual analysis of the figure 2.10 shows that during the first phase, bees mostly flew perpendicular to the nest direction in all four flights, as indicated by the presence of peaks around 90 and -90 degrees. When looking at the figure 2.11, no such peak is detected indicating that the distribution of flight directions was more uniform during the second phase of the learning flights. The perpendicular direction may be a sign that the first phase has a specific function. Alternatively, perpendicular directions could just be a consequence of looping very close to the nest. A visual inspection of the same figures shows that the bees' directions during the first learning flight from the flower were

almost uniformly distributed. By the second flight, there was a small peak directly away from the flower which grew larger in flights 3 and 4. The same peak continued in the 2nd phase of flights from the flower. It was small in the first flight and grew in size across flights 2 to 4.

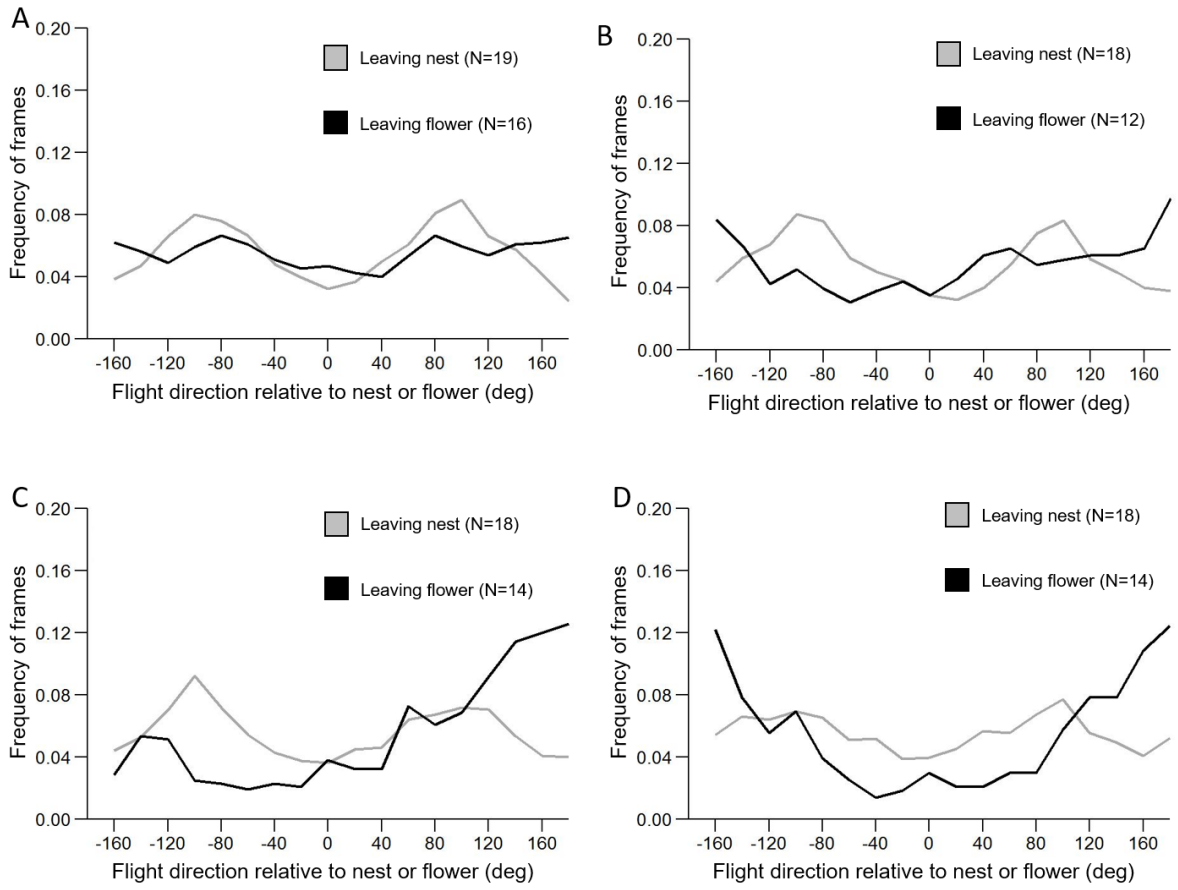


Figure 2.10. Frequency distribution of the bees' flight directions relative to nest or flower for the early part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°.

This pattern suggests that any learning that may occur during the second phase of the flight is limited to the first flight. In later flights, the bees are just flying away.

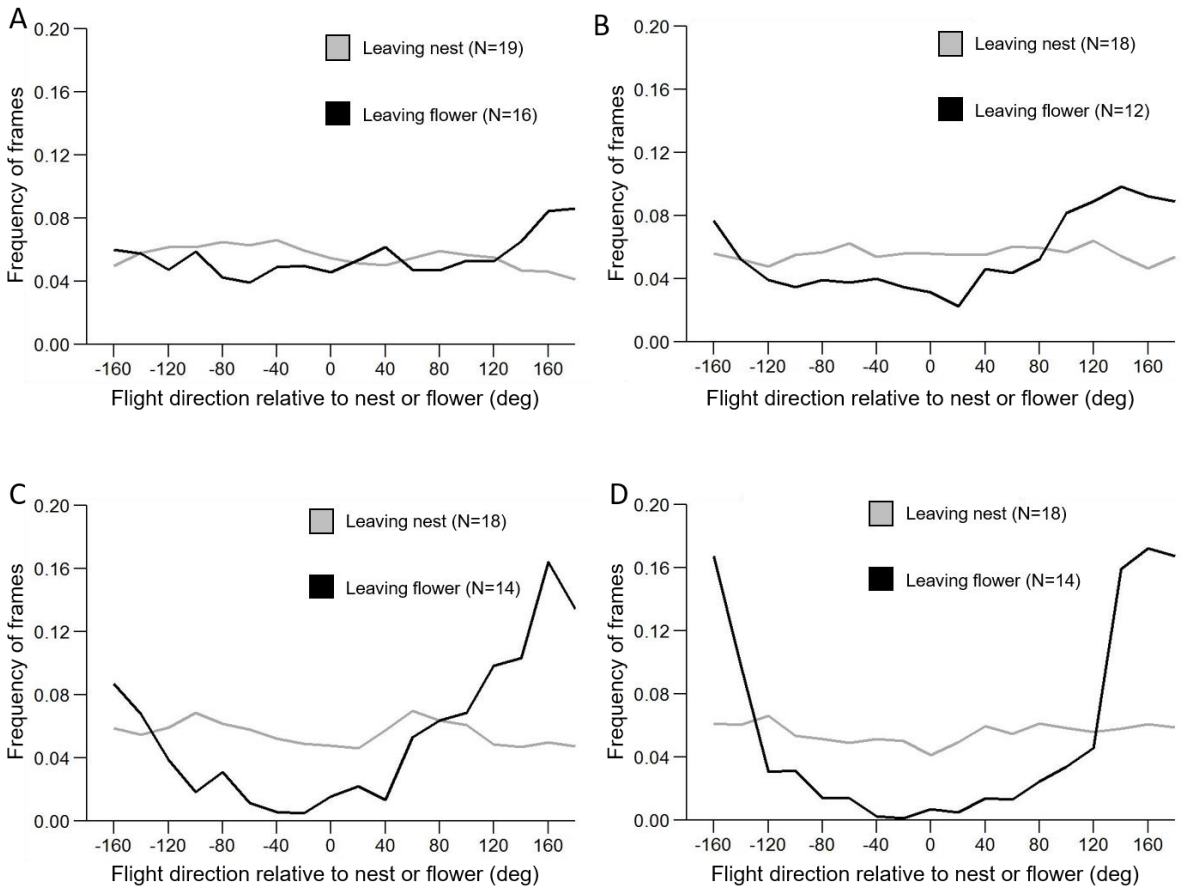


Figure 2.11. Frequency distribution of the bees' flight directions relative to the nest or flower for the late part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°.

The difference of flight patterns during the second phase from the nest and the flower becomes clear in the heat map of figure 2.12 containing the superimposed paths of all the bees on their first flights from the nest and flower. Looking at the figure 2.12, we can see that the flights from the nest cover a much larger area than those from the flower indicating that the bees survey and may learn about a larger area around the nest than the flower.

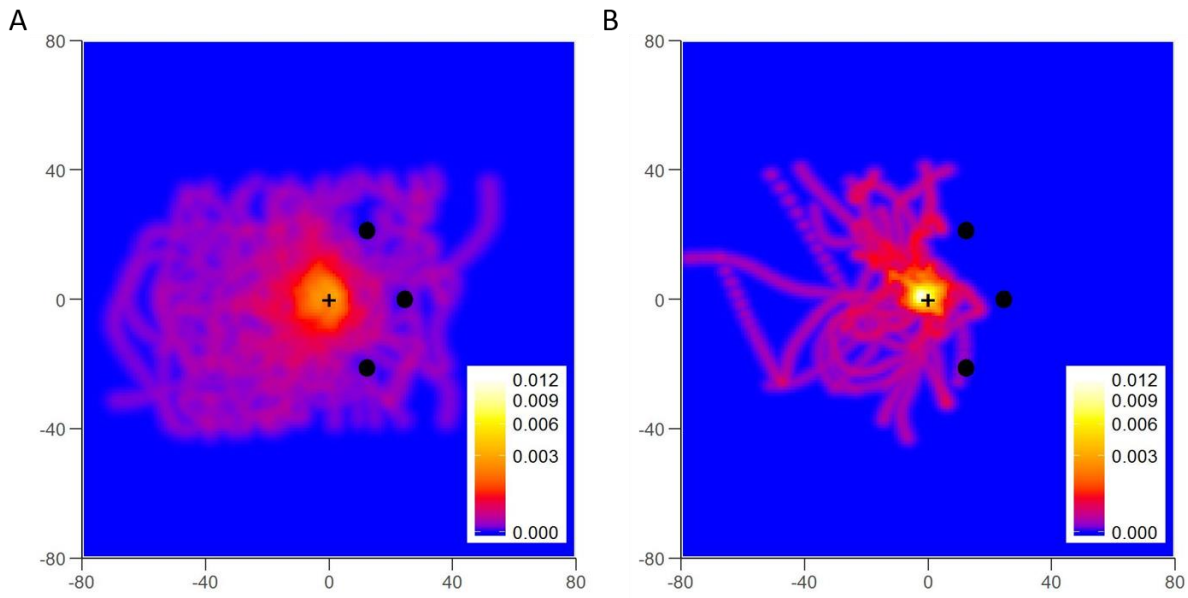


Figure 2.12. Heat maps of the kernel density estimations of the bees' position from the combined late phases of the first learning flights at the nest (A) and the flower (B). Each bee position was weighted in function of its time relative to the total duration of the flight (i.e. time of the frame/duration of the flight). The black circles represent the position of the cylinders and the cross is the position of the nest or the flower.

The shorter duration and length of the flights at the flower than at the nest may indicate that the bees expend less effort to learn the location of the flower than the location of the nest. The next step in examining this possibility is to analyse how the bees look at the nest and flower.

2.3.3 Do the bees view the nest and the flower differently during their learning flights?

We started by analysing the compass direction in which the bees face during the two phases of the learning flights and found a striking difference between flights from the flower and

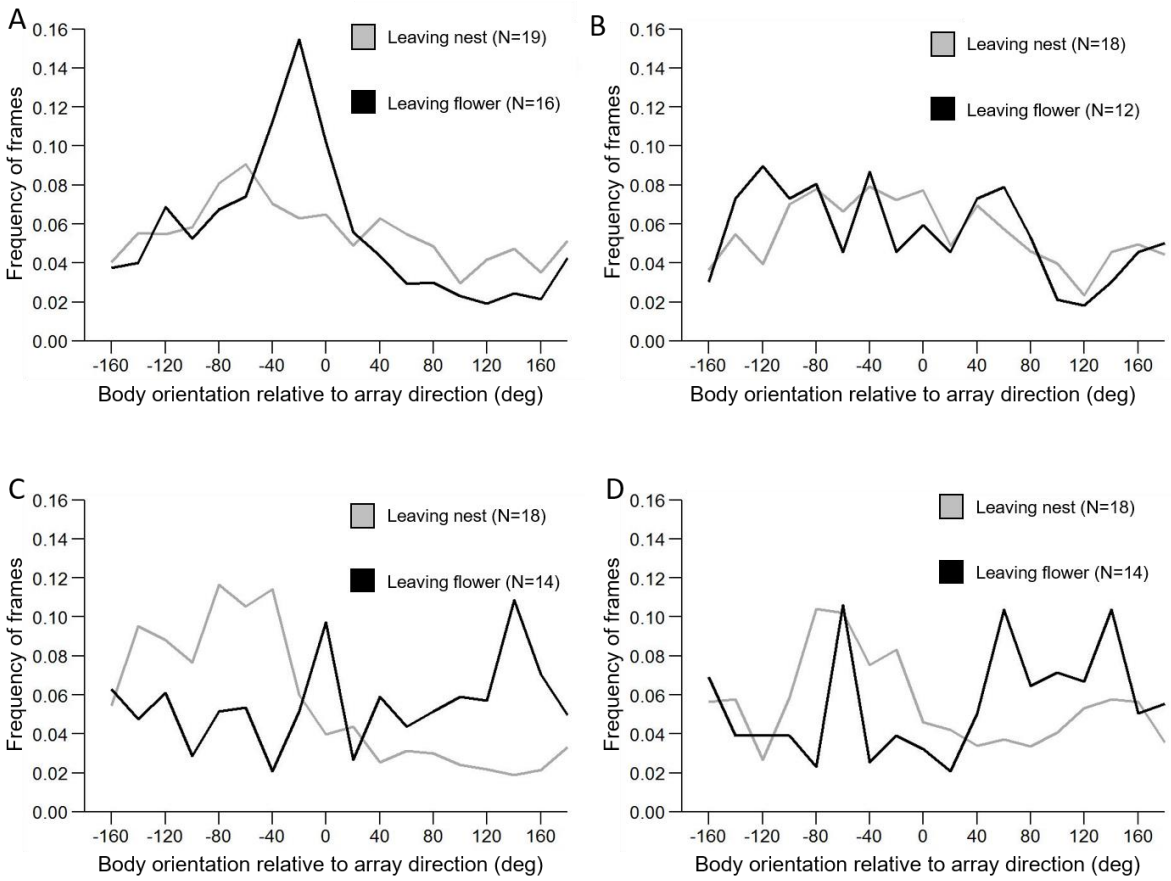


Figure 2.13. Frequency distribution of the bees' orientation relative to array-direction for the early part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°. Mean orientations and vector amplitude at the nest are: flight 1: mean=-49.98deg, rho=0.16; flight 2: mean=-36.27deg, rho=0.17; flight 3: mean=-79.30deg, rho= 0.39; flight 4: mean=-72.42deg, rho=0.16. Mean orientations and vector amplitude at the flower are: flight 1: mean=-40.46deg, rho=0.38; flight 2: mean=-60.56deg, rho=0.16; flight 3: mean=132.61deg, rho= 0.11; flight 4: mean=119.56deg, rho=0.16.

from the nest. When visually analysing the figure 2.13, we can see that the during the first phase of the four flights from the nest, the orientations were shifted toward the negative values meaning that the bees were oriented roughly eastward of the cylinder array. With bees close to the nest, this direction might indicate some preference for looking at the right cylinder. The same analyses applied to the Figure 2.14 shows that in the second phase of

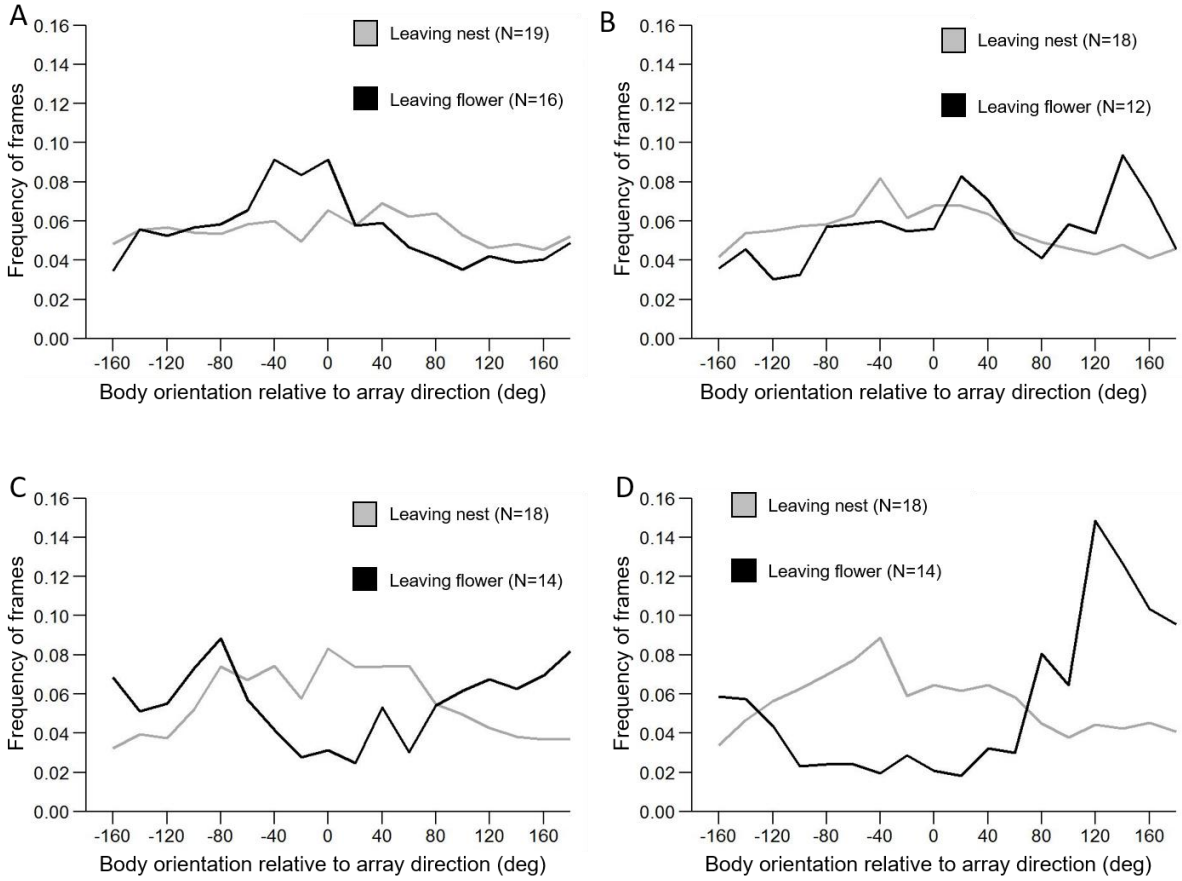


Figure 2.14. Frequency distribution of the bees' orientation relative to array-direction for the late part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 20°. Mean orientations and vector amplitude at the nest are: flight 1: mean=5.04deg, rho=0.06; flight 2: mean=-25.10deg, rho=0.12; flight 3: mean=-1.67deg, rho= 0.19; flight 4: mean=-34.49deg, rho=0.16. Mean orientations and vector amplitude at the flower are: flight 1: mean=-33.38deg, rho=0.19; flight 2: mean=59.17deg, rho=0.09; flight 3: mean=-166.09deg, rho= 0.17; flight 4: mean=142.89deg, rho=0.44.

flights from the nest, orientations were distributed more uniformly though preferentially in the direction of the cylinder array as indicated by the slightly higher values around 0 degrees. The visual analyses of the figure 2.13 also shows that in the first phase of the 1st flight from the flower, the orientation distribution present a strong peak at around -20 degrees. This

means that the bees were strongly oriented northeastward relative to the cylinder array, in the direction of the central cylinder. In later flights the distribution became more uniform with several small peaks. The peak toward the array continued into the second phase of the first flight (Figure 2.14). Over later flights a peak developed to the opposite direction of the cylinder array, in the bees' departure direction. The large differences in viewing directions between flower and nest on the first learning flight suggest that the bees may learn different features of the locations of a flower and their nest.

When visually inspecting the distributions of orientations relative to the nest and the flower, we can see that the differences in the bees' compass orientation during the first flights from the nest and flower were not reflected in their looking directions relative to the nest or flower. In the Figure 2.15A and 2.16A, the strong peaks around 0 degrees indicate that the bees were strongly oriented towards both nest and flower during the first and the second phases of their first flight . On the second flights, the peaks towards the nest and flower remained prominent in the first phase, but in the second phase the peak was only evident in flights from the nest (Figure 2.15B and 2.16B). In the third and fourth flights the bees continued to face the nest, but were oriented away from the flower as shown by the peaks around 180 degrees. To summarise these differences, first, bees faced the flower while looking to the central cylinder direction (roughly North), but looked at the nest over a wider range of directions and, second, across the four flights, bees were more persistent in facing the nest than in facing the flower.

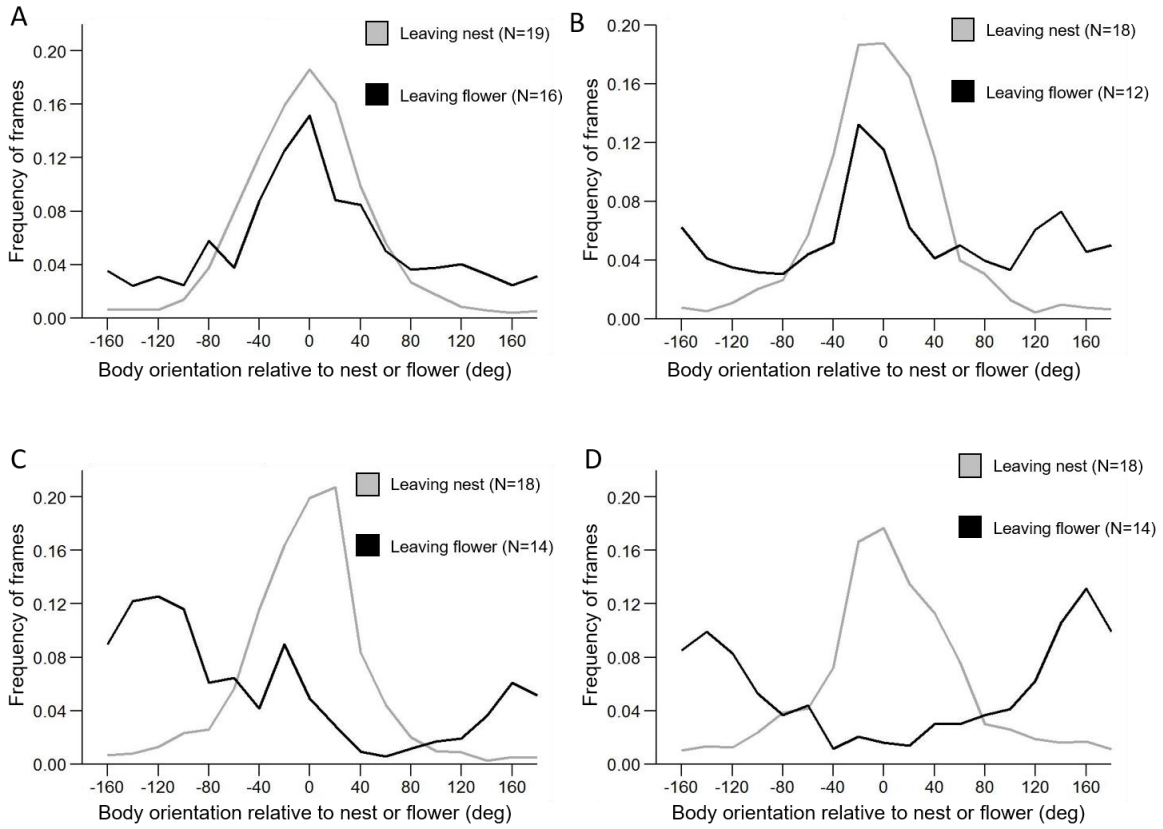


Figure 2.15. Frequency distribution of the bees' orientation relative to nest or flower for the early part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Mean orientations and vector amplitude toward the nest are: flight 1: mean=-3.31deg, rho=0.70; flight 2: mean=-2.53deg, rho=0.70; flight 3: mean=-3.28deg, rho=0.72; flight 4: mean=4.75deg, rho=0.60. Mean orientations and vector amplitude towards the flower are: flight 1: mean=9.84deg, rho=0.17; flight 2: mean=10.34deg, rho=0.13; flight 3: mean=-112.33deg, rho=0.43; flight 4: mean=-178.02deg, rho=0.42. Bin width is 20°.

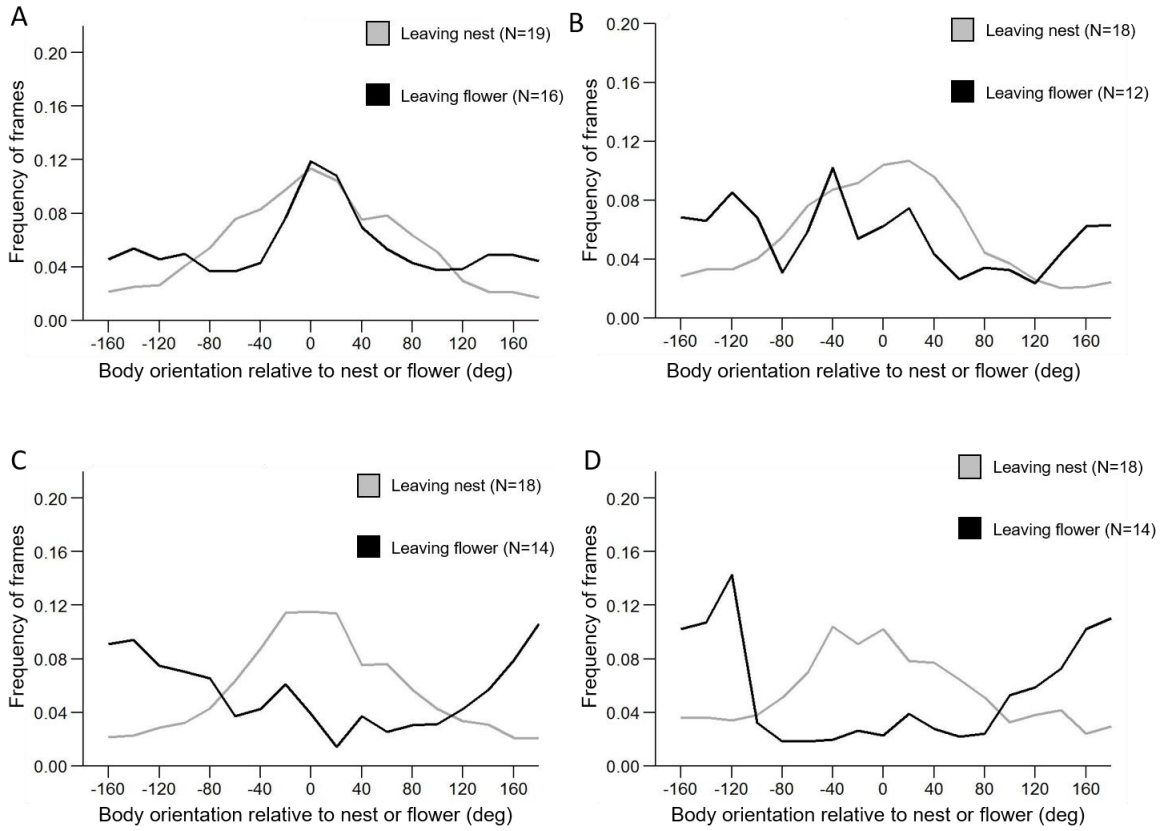


Figure 2.16. Frequency distribution of the bees' orientation relative to nest or flower for the late part of the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Mean orientations and vector amplitude towards the nest are: flight 1: mean=2.98deg, rho=0.39; flight 2: mean=-3.05deg, rho=0.37; flight 3: mean=5.25deg, rho=0.40; flight 4: mean=-5.63deg, rho=0.29. Mean orientations and vector amplitude towards the flower are: flight 1: mean=9.84deg, rho=0.17; flight 2: mean=-88.93deg, rho=0.15; flight 3: mean=-146.67deg, rho=0.29; flight 4: mean=-171.85deg, rho=0.41. Bin width is 20°.

2.3.4 Fixations of flower and nest

Very similar trends are found if, instead of analysing single frames, we examine fixations that we define as moments in which bees maintain the nest or flower in a constant position on their retina for at least 80 ms (see Chapter 1). We visually examined the distribution of the

fixations binned according to compass direction (Figure 2.17). The slightly higher values around -40 degrees show that fixations during all four learning flights at the nest were broadly distributed in a predominantly north-easterly direction relative to the cylinder array. Fixations on the first learning flight from the flower were also oriented in this direction as shown by the peak in the distribution. The absence of the same peak indicates that directions

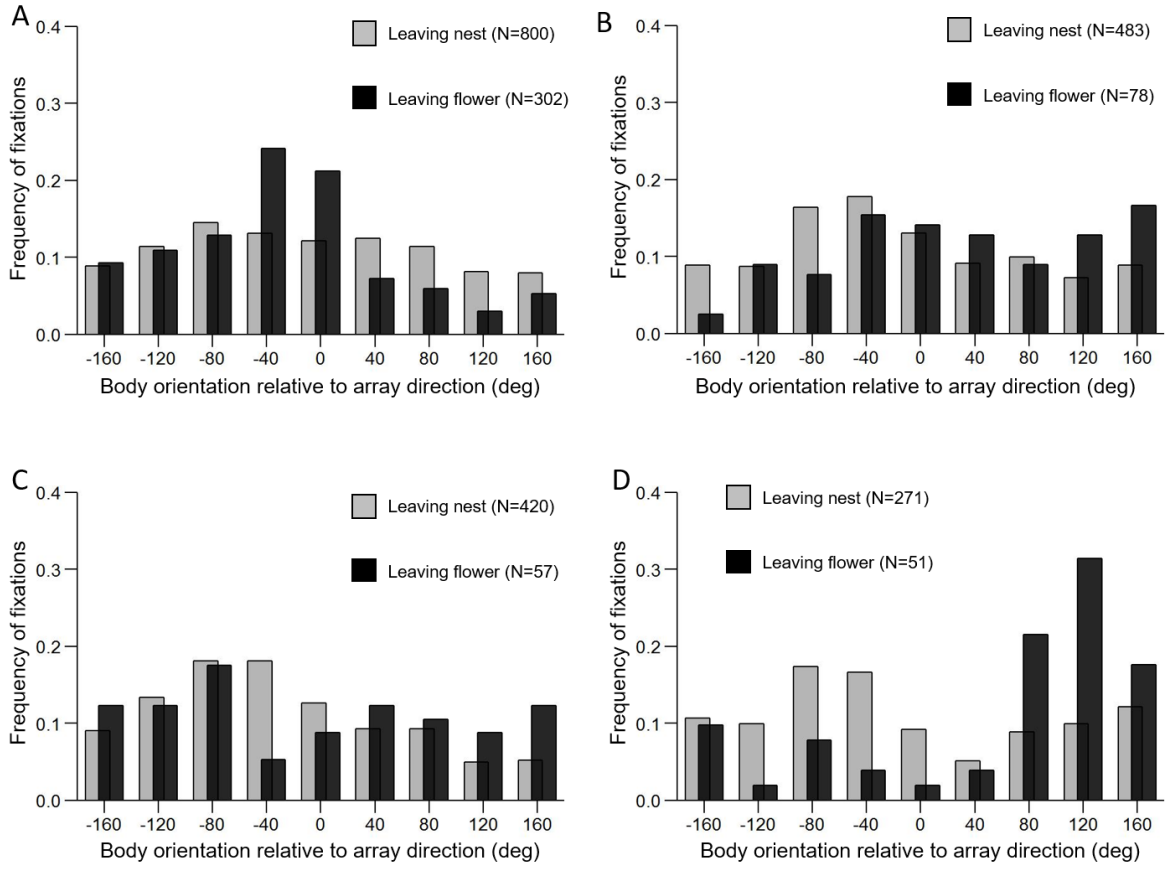


Figure 2.17. Frequency distribution of the bees' fixations orientation relative to array-direction during the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 40°.

were distributed more broadly on the second and third flights. On the fourth flight from the flower, the distribution presented a strong peak at 120 degrees meaning that the fixations were in the direction opposite to the array, in the departure direction.

The visual inspection of the distribution of the fixations binned relative to the nest or flower shows that during the first learning flights at both nest and flower, the fixation peaked in direction of the two goals (Figure 2.18). On flights two to four from the nest, the fixations were also directed towards the nest. The peak around 0 degrees during the first flight at the flower and the shift of this peak toward the 180 opposite orientation over the successive flights show that the bees only had a strong tendency to fixate the flower during their first learning flight. Thereafter, the directions of fixations changed progressively. By the fourth learning flight most fixations were in the opposite direction corresponding to bees flying away from the flower.

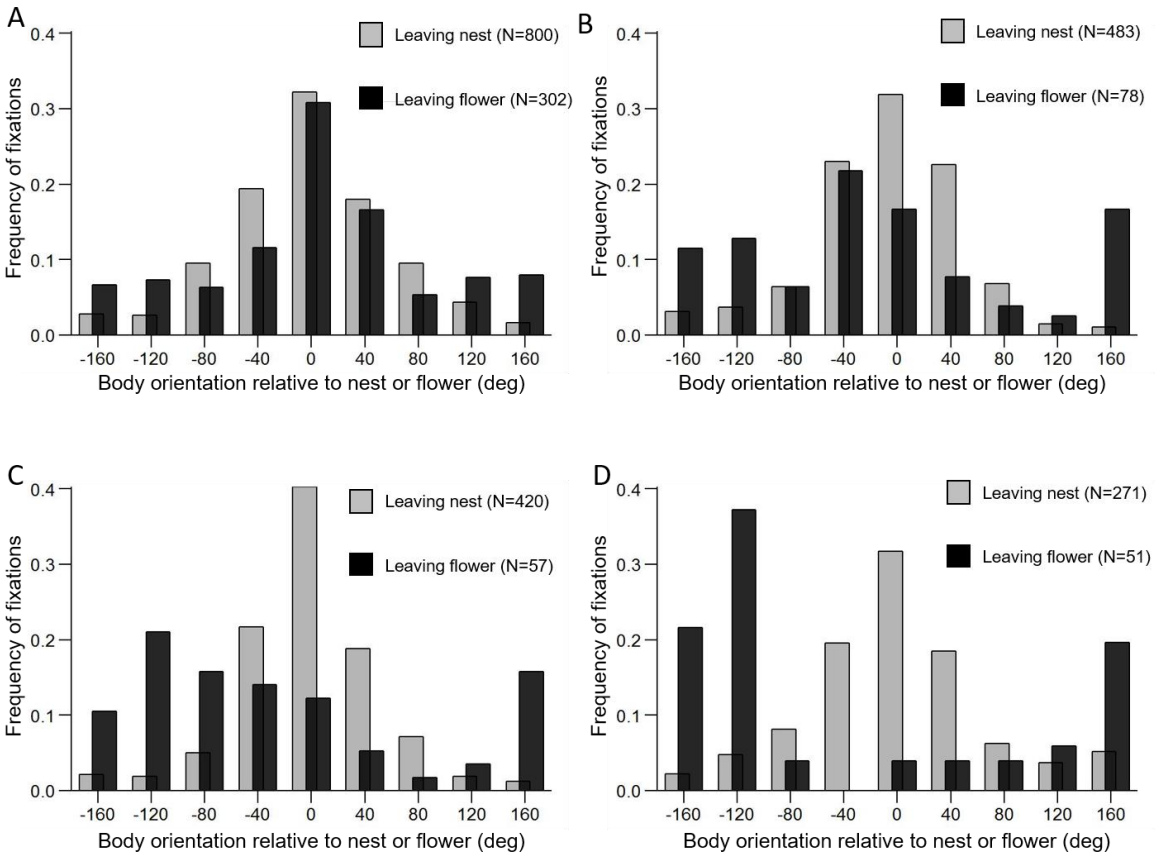


Figure 2.18. Frequency distribution of the bees' fixations orientation relative to nest or flower during the first (A), second (B), third (C) and fourth (D) learning flights at the nest (grey) or flower (black) location. Bin width is 40°.

Visual examination of the Figure 2.19 informs us that a characteristic common to all the learning flights at the nest and the feeder is that the great majority of nest and flower fixations (±20deg) occurred within 10 cms of that location. This clustering is in part because bees spend most time close to the nest and feeder (Figure 2.7A), but in part it is because the rate of fixations (ratio of the number of frames in fixations to the total number of frames within a bin) is higher in the early part of the flight as shown by the GEE model (Table 2.2) and visually confirmed by the graph presented in Figure 2.20.

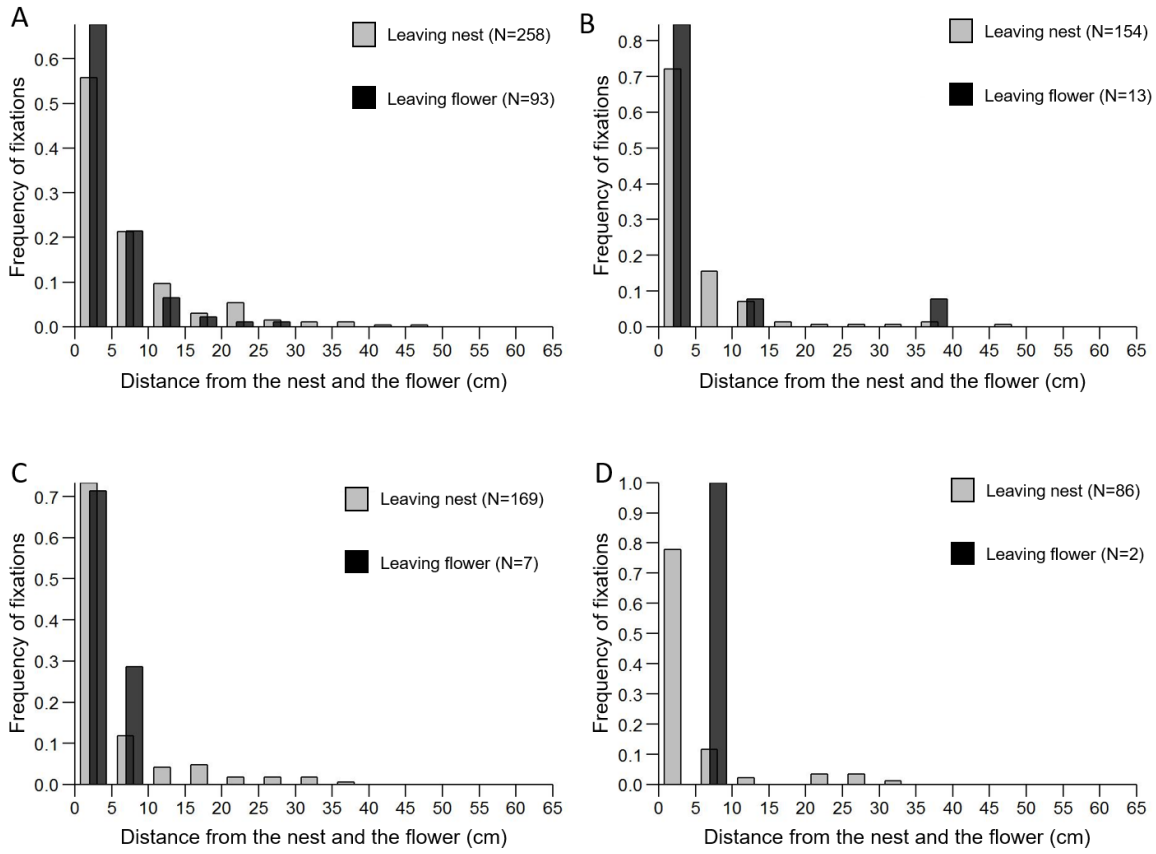


Figure 2.19. Normalised distribution of the facing fixations (±20°) depending on their distance from nest or flower during the first (A), second (B), third (C) and fourth (D) learning flights. The bars show the proportion of the fixations oriented toward the nest (in grey) or the flower (in black) that fall into each 5 cm distance bin.

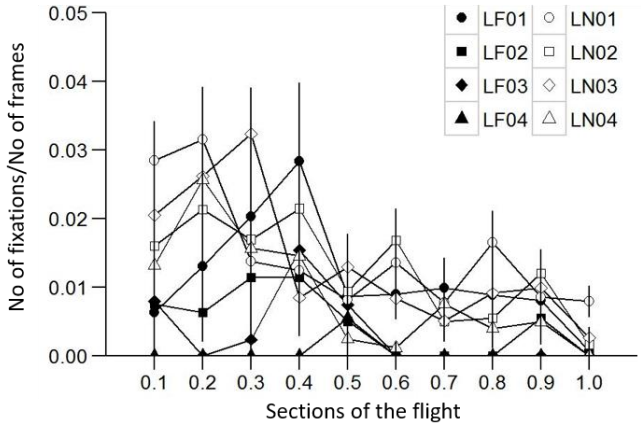


Figure 2.20. Mean rates of fixations centred on the goal (±20°) within regular section of the learning flights 1 to 4 at the nest (white symbols) or the flower (black symbols). For each bee, the number of fixations in each flight section is divided by the number of frames present in this flight section.

Table 2.2. GEE modelling examining the predictor influencing the bees' rates of fixation toward the goals (±20°) within each tenth of the learning flights (number of fixations in the flight section/number of frames in the flight section). Bees decreased their fixation rate during their flights as well as over trials. The learning flights at the nest presented higher fixation rated than the learning flight at the flower.

Dependent Variables	Predictors	Estimates	Standard Error	$\chi 2$	*df*	*P*
Fixation rate	Flight section	-5.20E-04	7.14E-05	53	1	**<0.001**
	Trial	-7.74E-04	1.56E-04	24.5	1	**<0.001**
	Goal Nest	3.56E-03	8.70E-04	16.8	1	**<0.001**

2.3.5 Do bees fixate the cylinders during learning flights?

In order to determine whether bees faced the cylinders more frequently than they faced in other directions, we computed the frequency of fixations towards 9 virtual cylinders, in

addition to the 3 real ones, placed at 24.5cm at 30^{o} intervals in a circle centred on the nest and flower (Figs. 2.19 and 2.20). To test whether there was a significant preponderance of real over virtual cylinder fixations, we computed for each flight of each bee the mean number of fixations toward the 3 real cylinders (Figure 2.21: -60, 0 and 60 degrees) and the mean number of fixations toward the 4 virtual cylinders flanking the real ones (Figure 2.20: -90, -30, 30 and 90 degrees). This calculation gave mean values per real and virtual cylinder for each flight. We could detect more fixations toward the real cylinder (median of the individual means 4.33, IQR=1.50) than toward the virtual ones (median of the individual means 3.50, IQR=2.50) in the first learning flight from the nest (Wilcoxon test, N=19, W=200, Z=-2.25, p=0.02). Similarly, there were significantly higher means of fixations of the real cylinders (median of the individual means 1.50, IQR=2.00) than the virtual ones (median of the individual means 0.50, IQR=1.06) during the first learning flight from the flower (Wilcoxon test, N=16, W=100, Z=-3.31, p=9e-04). But, in later flights from both the nest and the flower, the real cylinders did not attract more fixations than the flanking virtual ones. (2nd flight nest: Wilcoxon test, N=18, W=60, Z=-0.947, p=0.3; 2nd flight flower: Wilcoxon test, N=12, W=30, Z=-0.613, p=0.5; 3rd flight nest: Wilcoxon test, N=18, W=60, Z=-0.233, p=0.8; 3rd flight flower: Wilcoxon test, N=14, W=30, Z=-1.97, p=0.05; 4th flights nest: Wilcoxon test, N=18, W=40, Z=-1.66, p=0.1; 4th flight flower: Wilcoxon test, N=14, W=5, Z=0, p=1).

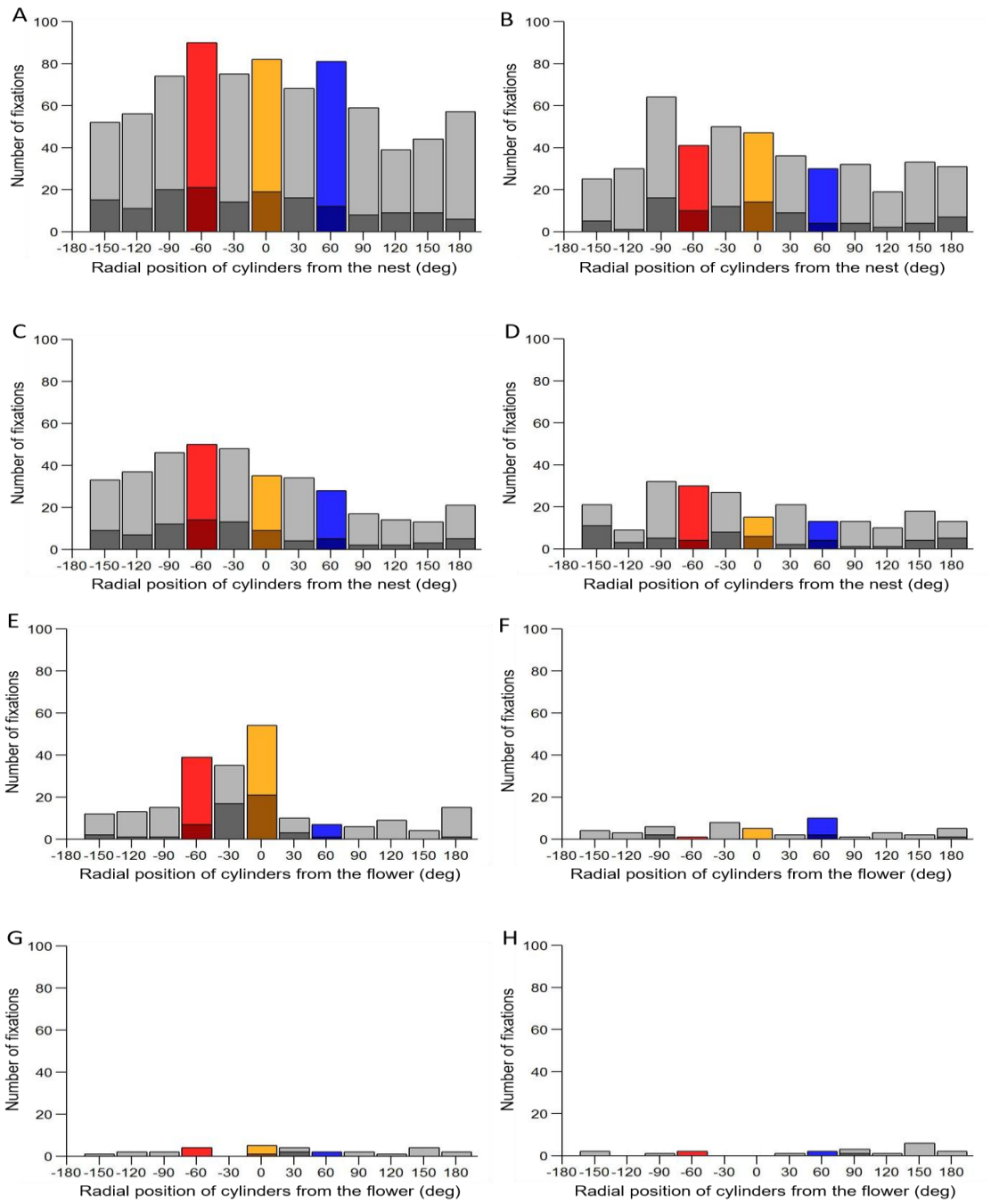
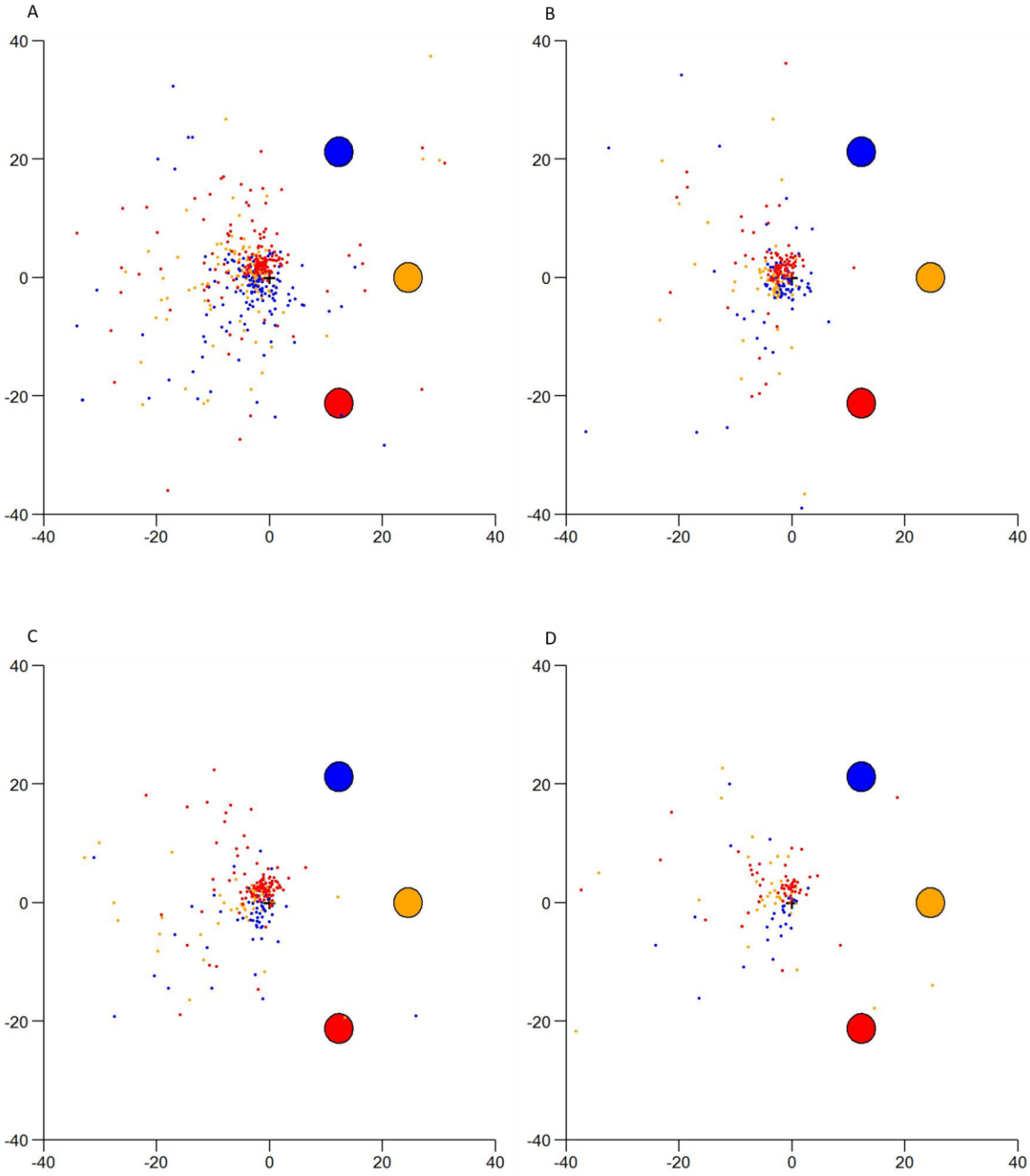


Figure 2.21. Number of fixations toward the real (coloured) and virtual (grey) cylinders (±10°) depending on their radial position around the nest during the first (A), second (B), third (C) and fourth learning flights (D) or around the flower during the first (E), second (F), third (G) and fourth learning flights (H). The darker bars indicate the number of the fixations that are also toward the nest or the flower (±10°).


A
B
C
D
40
20
0
-20
-40
-40
-20
0
20
40

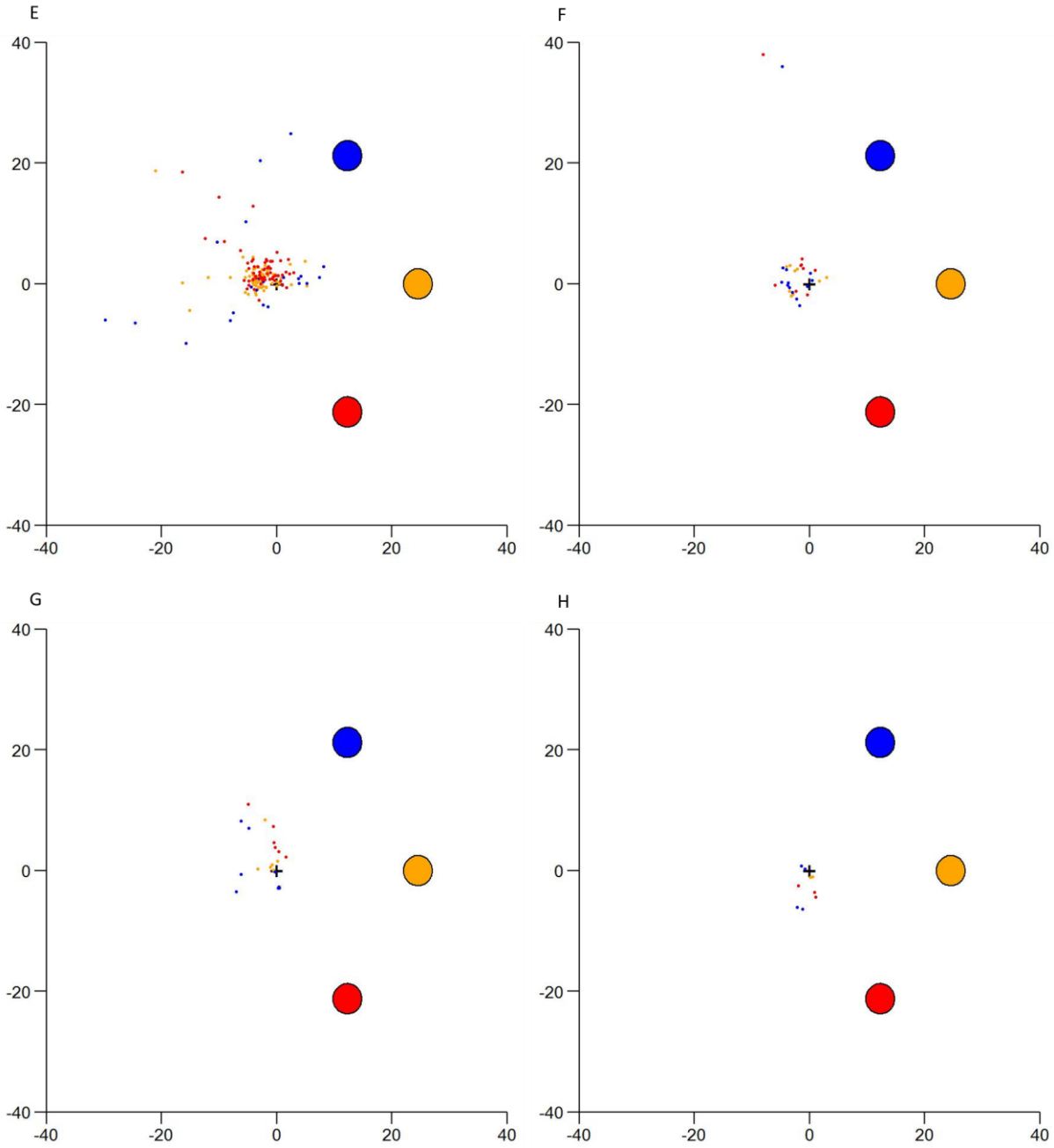


Figure 2.22. Bees' position during fixations toward the cylinders (±20°) during the first (A), second (B), third (C) and fourth (D) learning flights at the nest and the first (E), second (F), third (G) and fourth (H) learning flights at the flower location. Each of the points represent the position of a bee during the first frame of a fixation toward the left (in blue), central (in yellow), or right cylinder (in red). The corresponding cylinders' positions are shown by the large coloured circles. The nest or flower location is marked by a black cross.

A visual inspection of the figure 2.22 shows that, as it is the case with fixations of the nest or flower, the majority of fixations of the cylinders also occur close to the bee's departure point. Those that are more distant are mostly on side of the nest away from the cylinders. The cylinder fixation rates during the first learning flights from the nest and flower peak at different times during the normalised flights (Figure 2.23). On flights from the nest the fixation rate of the right cylinder peaks at the start of the flight followed soon by the central cylinder at a slightly lower fixation rate and still later the left cylinder at the lowest rate of the three. On flights from the flower only the central cylinder attracts appreciable fixations with a broad peak in the fixation rate about a third of the way through the flight. These patterns suggest a viewing strategy that differs temporally and spatially across nest and feeder flights.

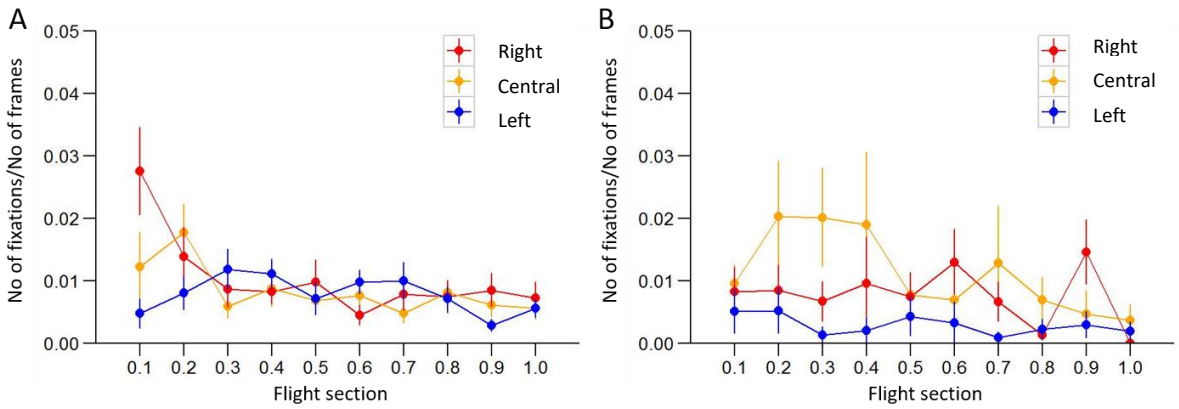
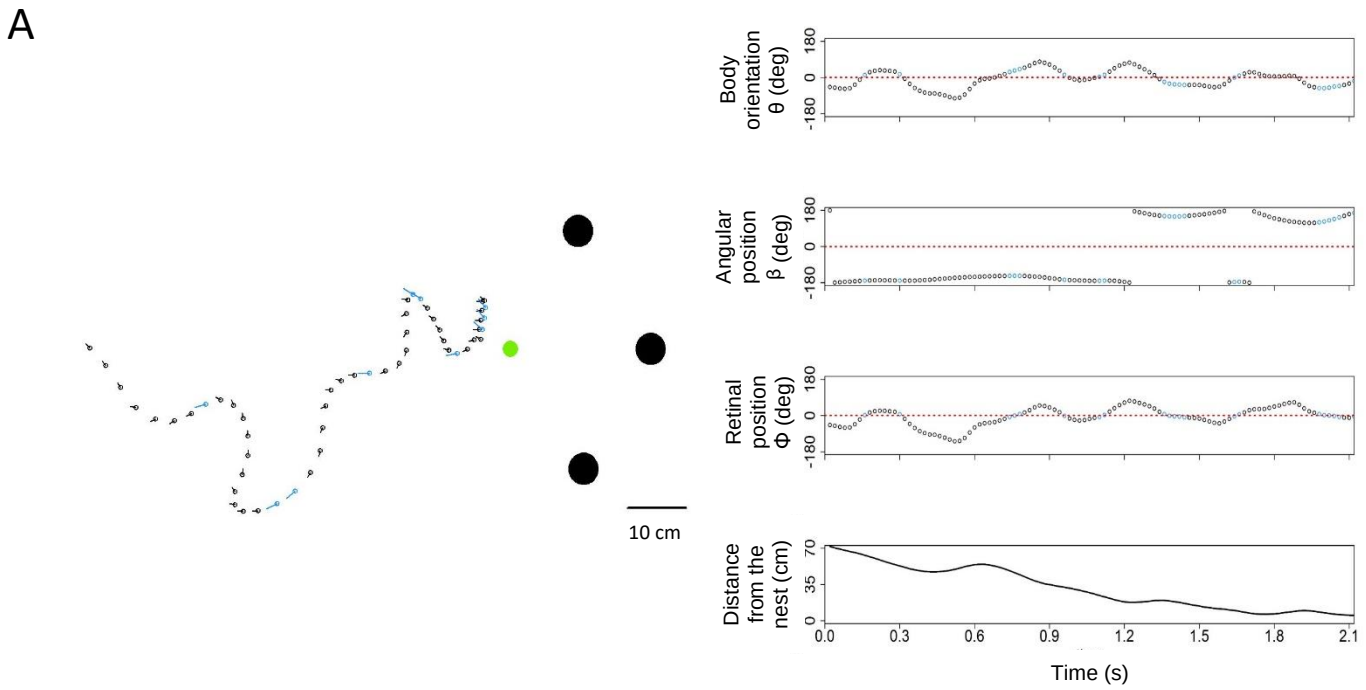
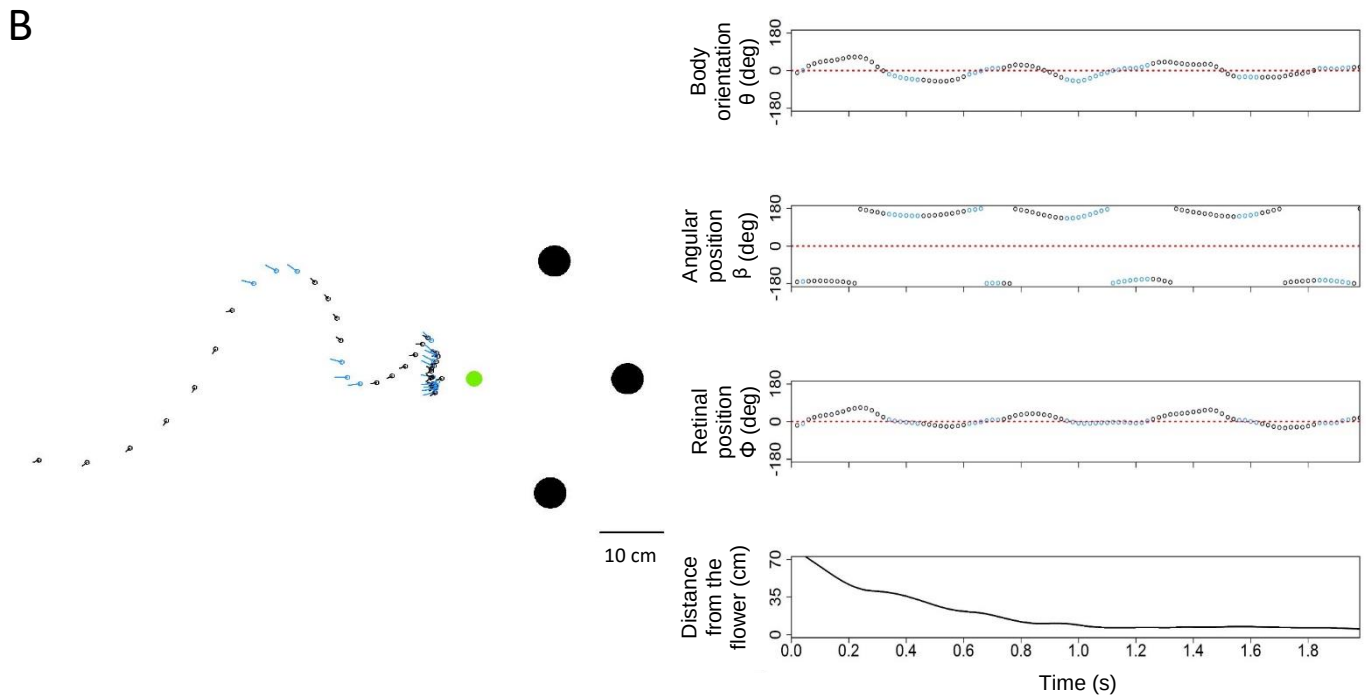


Figure 2.23. Mean rates of fixations centred on the cylinders (±20°) within regular section of the first learning flights at the nest (A) or the flower (B). For each bee, the number of fixations in each flight section is divided by the number of frames present in this flight section.

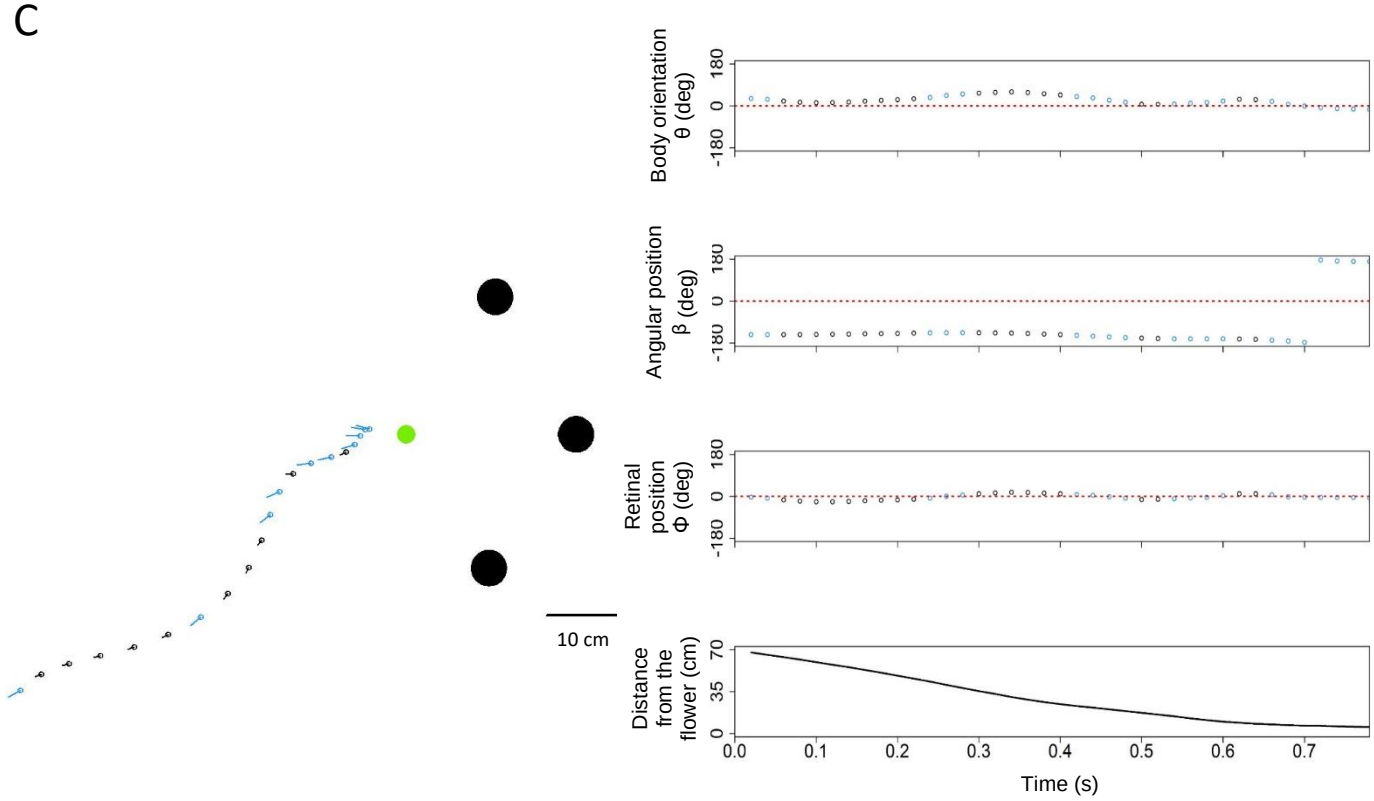
2.3.6 Return flights

Bumblebees returning to their nest typically approach it while flying in a characteristic zigzag pattern (e.g. Figure 2.24A) (Philippides *et al.*, 2013). The example bee in figure 2.24 illustrates that the same zigzag pattern occurs during approaches to the flower (Figure 2.24B, C, E) with the bee tending to face the goal at the extrema of the zigzags (cf. Philippides *et al.*, 2013). Approaches to the flower and the nest are also similar in the way that the bee slows down during the approach, often hesitating, circling or hovering close to the goal before landing. Surprisingly, there are no striking differences between the 1st and 4th returns. Indeed, when visually examining the trajectories, the bee's first approach to the flower, before it has performed any learning flight there (Figure 2.24B, RF00) appears much the same as its fourth return. It should be noted though that the bee did perform a learning flight when leaving a similar flower on the training table.

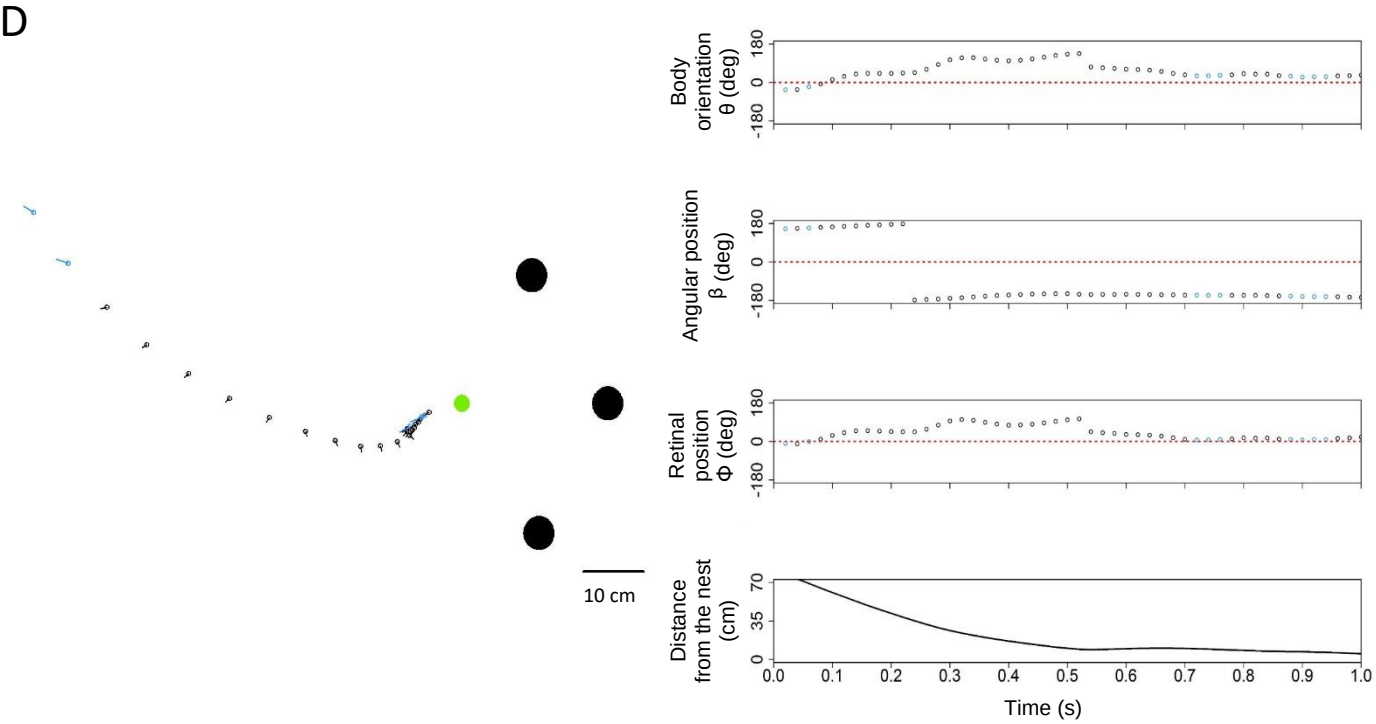

A
Body orientation θ (deg)
Angular position β (deg)
Retinal position Φ (deg)
Distance from the nest (cm)
180
0
-180
70
35
0
0.0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
Time (s)
10 cm


B
Body orientation θ (deg)
Angular position β (deg)
Retinal position Φ (deg)
Distance from the flower (cm)
180
0
-180
70
35
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Time (s)
10 cm

C


Body orientation θ (deg)
Angular position β (deg)
Retinal position Φ (deg)
Distance from the flower (cm)
10 cm
Time (s)

D


Body orientation θ (deg)
Angular position β (deg)
Retinal position Φ (deg)
Distance from the nest (cm)
10 cm
Time (s)

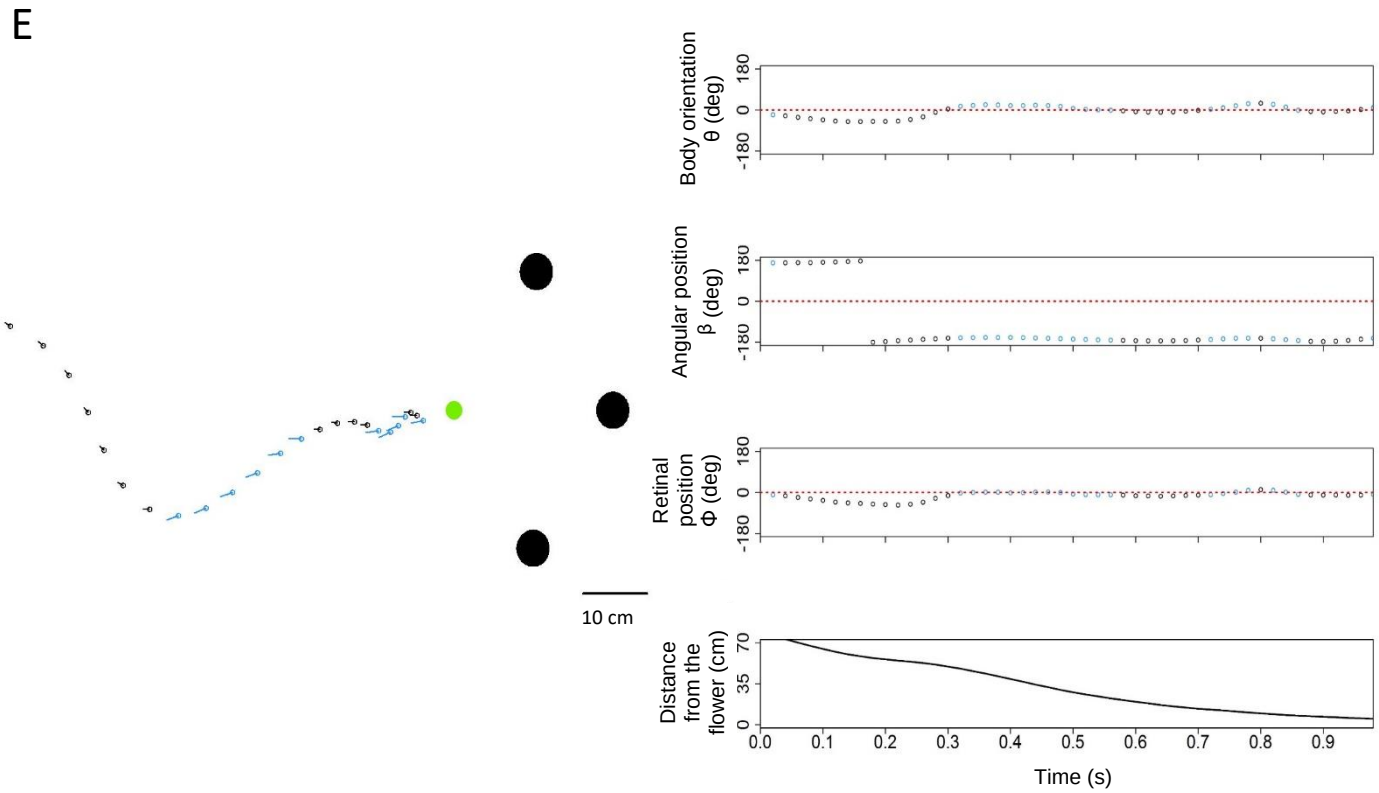


Figure 2.24. On the left: Top view of the trajectory of the bee FB4 during her first return at the nest (A) as well as her first approach (B) and first return to the flower (C), and the fourth return to the nest (D) and the flower (E). The large black filled circles represent the position of the cylinders. The green circle shows the location of the nest or the flower. The empty circles are the positions of the bee plotted every two frames and the tails represent the orientation of the bee. The blue circles represent the frames on which the bee is oriented toward the nest or the flower (± 10°). On the right: Bees' cardinal orientation, orientation relative to nest or flower, retinal position of the nest or flower and distance from the nest or flower during the learning flights.

The lack of any obvious improvement in the duration or length of the approach to the nest or flower is also seen in the plots of figure 2.25A and Table 2.3.1. The time taken to approach the nest (from 26cm away to 6cm) did not differ significantly (Wilcoxon test, N=15, W=90, Z=-0.521, p=0.6) between the first returns (median 0.62s, IQR=1.09) and the fourth returns (median 0.72s, IQR=0.25). The first returns (median 0.44s, IQR=0.14) and the fourth returns (median 0.43s, IQR=0.10) to the flower were also of similar durations (Wilcoxon test, N=14, W=50, Z=-0.09, p=0.9). But there are some differences between returns to the nest and the flower. The bees took longer to approach the nest than to approach the flower during both their first foraging trip (Wilcoxon test, N=15, V=20, Z=-1.99, p=0.05) and their fourth foraging trip (Wilcoxon test, N=13, W=10, Z=-2.41, p=0.02). This difference emerges gradually during the approaches and becomes obvious when the bees are 15 to 10cm from their goal (Figure 2.25A). Some hesitation very close (6 cm) to the flower is also seen in the bees' first approach to it (RF00).

Table 2.3. GEE modelling examining the influence of various predictors on several return flights parameters. Entry 1: The flight duration did not vary over trials but the return flights at the nest were longer than the return flights at the flower. Entry 2: The bees' mean ground speed when crossing radiuses of decreasing distances from the goal. Bees' speed decreased with their distance from the nest and the flower. The bees' ground speed did not vary over trial or between the two locations. Entry 3: The trajectory length of the return flights did not change over trial but were longer at the nest than at the flower location.

	Dependent Variables	Predictors	Estimates	Standard Error	$\chi 2$	*df*	*P*
1	Duration	Trial	-0.052	0.05	1.06	1	0.3
		Goal					
		Nest	0.412	0.078	28.2	1	**<0.001**
2	Speed	Radius	2.767	0.171	261	1	**<0.001**
		Trial	-2.655	1.865	2.02	1	0.15
		Goal					
		Nest	-4.716	5.928	0.63	1	0.43
3	Trajectory length	Trial	-2.18	2.1	1.09	1	0.3
		Goal					
		Nest	15.16	3.54	18.4	1	**<0.001**

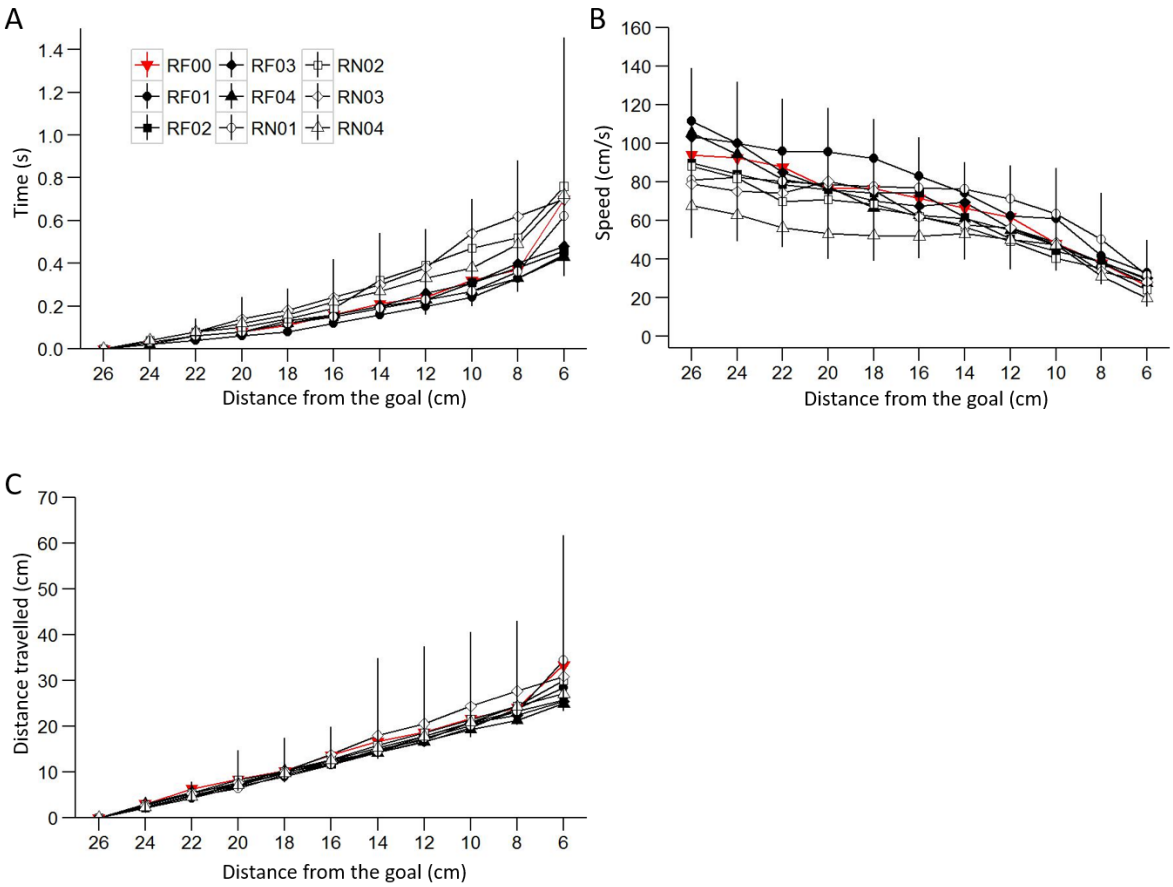


Figure 2.25. A: Median times taken by the bees before to cross different distances from the nest or the flower during the return flights 1 to 4 at the nest (white symbols) and at the flower location (black symbols), and the first approach of the flower (red symbols). The times are measured starting from the first crossing of the 26 cm distance to before the bees crossed a circle of a given radius to a minimum of 6 cm. B: Medians of each bee's mean speed when crossing different distances from the nest or the flower for the first time ± 2 frames. C: Median trajectory length flown by the bees before to cross different distances from the nest or the flower. Here, and later in the thesis, the first approach of the flower is called RF00, the return flights to the flower 1 to 4 are called RF01, RF02, RF03 and RF04. The return flights to the nest 1 to 4 are called RN01, RN02, RN03 and RN04.

Trajectory lengths also differed between approaches to the nest and flower, with those to the nest being significantly longer than those to the flower (Figure 2.25C; table 2.3.3). The median trajectory-length of the first return to the nest was 34.41cm, IQR=36.86 compared with 28.34cm, IQR=6.56 for that of the flower (Wilcoxon test, N=15, W=20, Z=-2.56, p=0.01). Median lengths of the fourth returns to the nest (27.07cm, IQR=9.92) and flower (24.91cm, IQR=3.06) also differed (Wilcoxon test, N=13, W=20, Z=-1.99, p=0.05). But the first and fourth returns to the nest were of similar length (Wilcoxon test, N=17, W=100, Z=-1.02, p=0.3) as were those to the flower (Wilcoxon test, N=14, W=80, Z=-1.48, p=0.1). It seems that, as in learning flights at the nest, the bees may survey the surroundings in more detail on their approach to the nest than they do on returns to the flower.

As is also the case in learning flights, there was no detectable difference in the flight speed of approaches to the nest and flower. Both the GEE model (table 2.3.2) and a visual inspection of the figure 2.25B show that the speed decreased during all approaches to the nest and the flower, including RF00, following a similar profile (table 2.3.2, Figure 2.25B). Some inaccuracies in these values arise because height also decreased, particularly at the start of the approach.

2.3.7 Approach and viewing directions on return flights

Bees tend to approach the nest and flower from a direction in which the array of cylinders lies beyond the goal, so enabling the bees to view the array throughout the approach (cf. Zeil 1993b). This direction to some degree mirrors roughly the bees' viewing directions during the first phase of the first learning flights from the nest and flower (Figs. 2.11A, 2.13A), as might be expected for learning flights that are adapted to assist view-based navigation.

One consistent difference between returns to the nest and flower that comes out of the data described in this section is that returns to the flower are more focussed in their direction than are returns to the nest (as shown in the previous section). These differences are likely to occur when the bees are near the goal, where differences in the lengths and durations of the nest and flower trajectories are greatest (Figure 2.25) and again suggest that bees are surveying the nest surroundings more widely on returns to the nest than the flower.

When visually examining the figure 2.26, we can see that the direction of bees returning to the nest is scattered but roughly aligned with the direction from the nest to the central cylinder. The directions towards the flower were similar but less scattered as indicated by the higher maximum frequencies. In contrast to approaches to the nest, the direction peaks in returns to the flower become sharper over the successive flight which indicates some improvement in consistency occurs over the bees' five approaches to the flower. The improvement may be a consequence of route learning during the five visits, since the nest, flower and central cylinder are all on the same line (Figure 2.5).

The greater consistency in the bees' compass direction to the flower compared with the nest was also apparent in their flight directions relative to these goals. A visual analysis of the figure 2.27 shows shaper peaks around 0 degree for the returns at the flower than for the returns at the nest. This element indicates that at the nest the bees' trajectories were less well directed toward the goal than were their trajectories to the flower.

The bees' body orientation on their returns to the nest and flower was also roughly toward the array as indicated by a visual inspection of the figure 2.28. Again, the taller peaks of orientations during the returns at the flower demonstrate that the tendency was stronger during returns to the flower than on returns to the nest. The figure 2.29 also shows that taller peaks for the five approaches of the flower than for the returns at the nest which indicate that

the body orientation measured relative to the goal was also aimed more precisely at the flower than at the nest, even before the first learning flight from the flower (RF00).

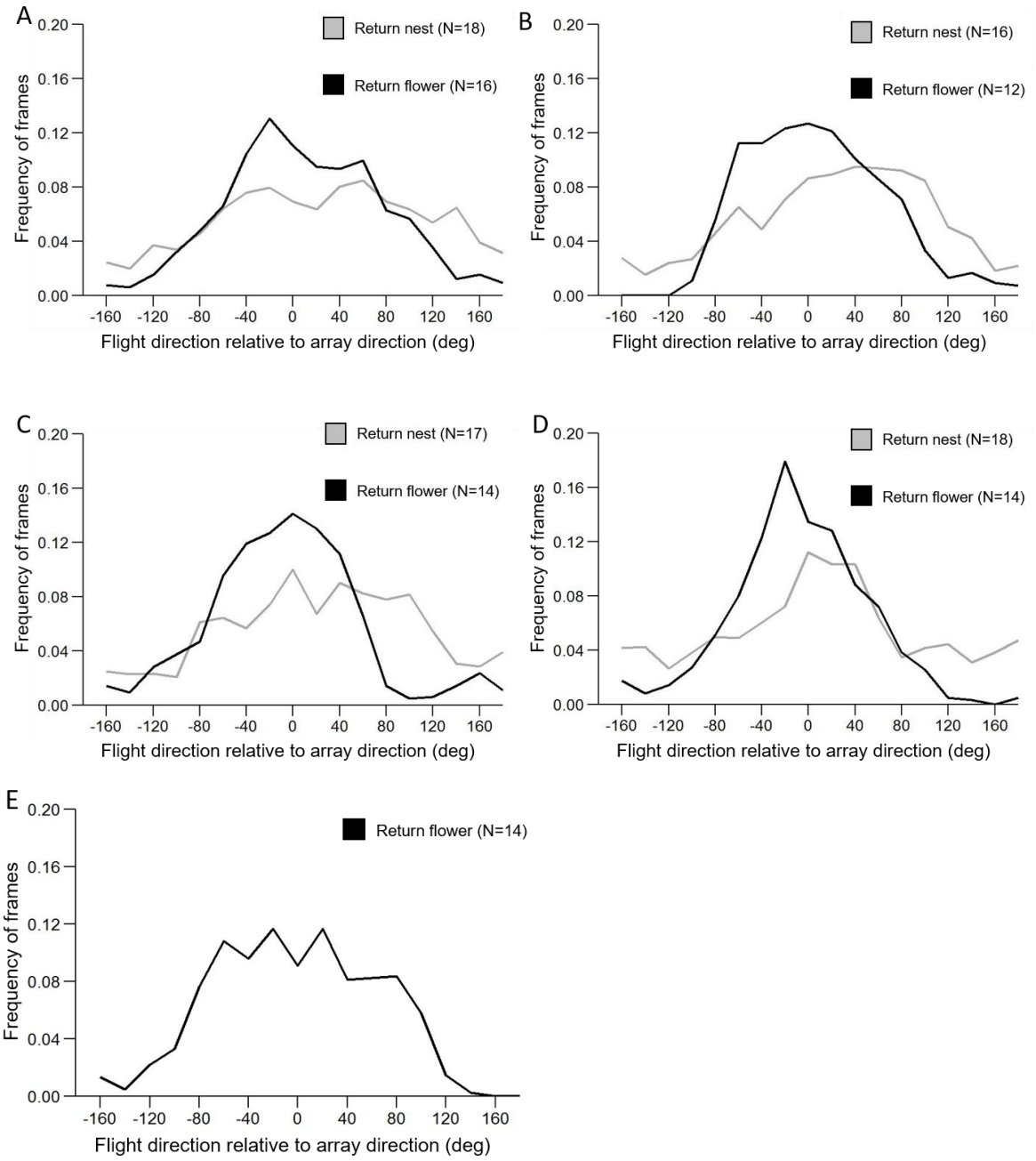


Figure 2.26. Frequency distribution of the bees' flight directions relative to orientation of the cylinder array for the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience (E). Mean direction to nest: return 1: mean=30.6deg, rho=0.23; return 2: mean=35.2deg, rho=0.35; return 3: mean=30deg, rho=0.30; return 4: mean=10.9deg, rho=0.25. Mean direction to flower: return 0: mean=-1.52deg, rho=0.51; return 1: mean=8.32deg, rho=0.50; return 2: mean=2.99deg, rho=0.60; return 3: mean=-10.8deg, rho=0.58; return 4: mean=-7.06deg, rho=0.64. Bin width is 20°.

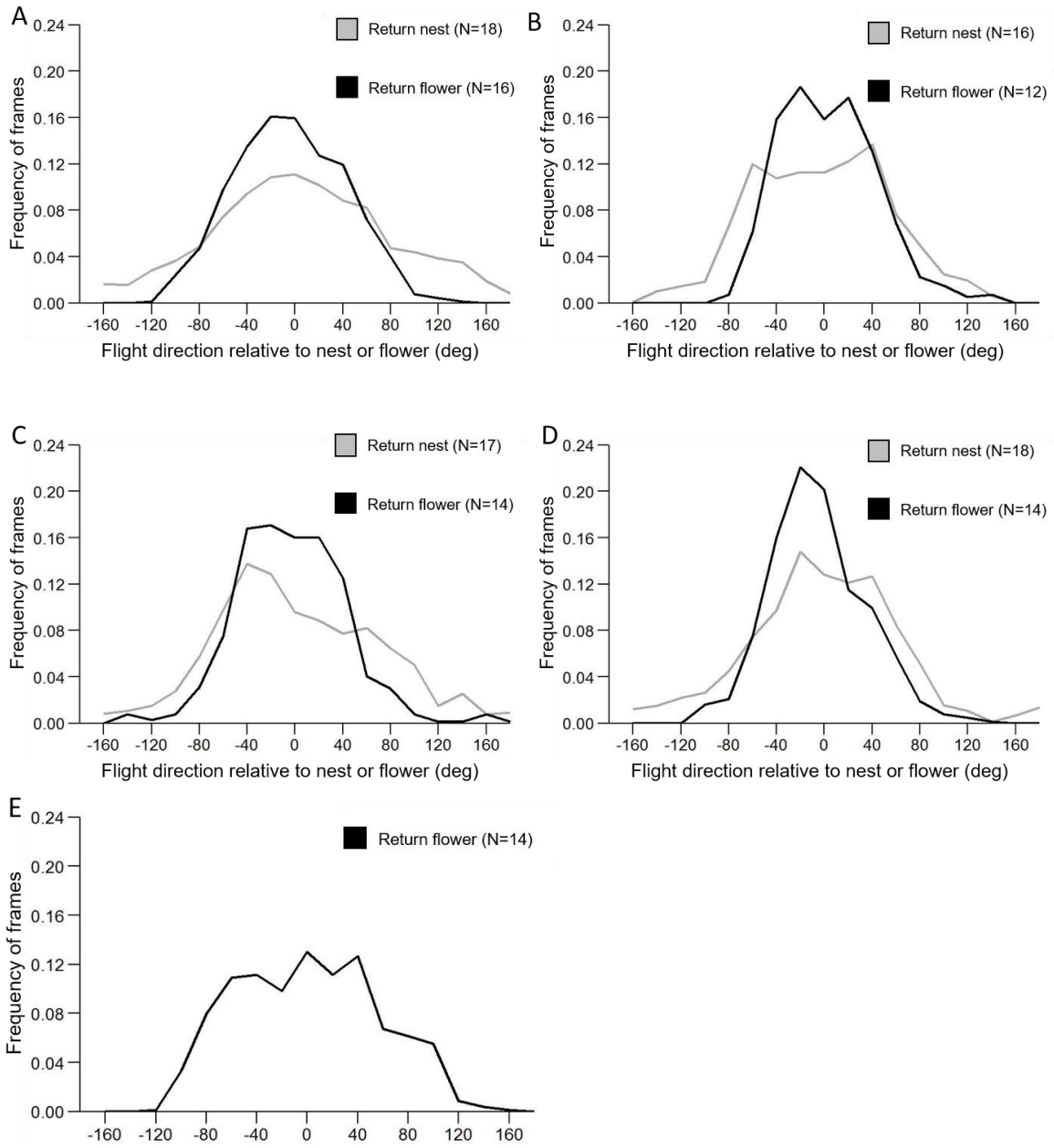


Figure 2.27. Frequency distribution of the bees' flight directions relative to the goal for the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience (E) Direction relative to nest: return 1: mean=3.6deg, rho=0.42; return 2: mean=-2.17deg, rho=0.61; return 3: mean=-3.09deg, rho=0.50 ; return 4: mean=-0.69deg, rho=0.58. Direction relative to flower: return 0: mean=-0.91deg, rho=0.61; return 1: mean=-4.99deg, rho=0.72; return 2: mean=1.99deg, rho=0.78; return 3: mean=-4.83deg, rho=0.75; return 4: mean=-6.84deg, rho=0.79. Bin width is 20°.

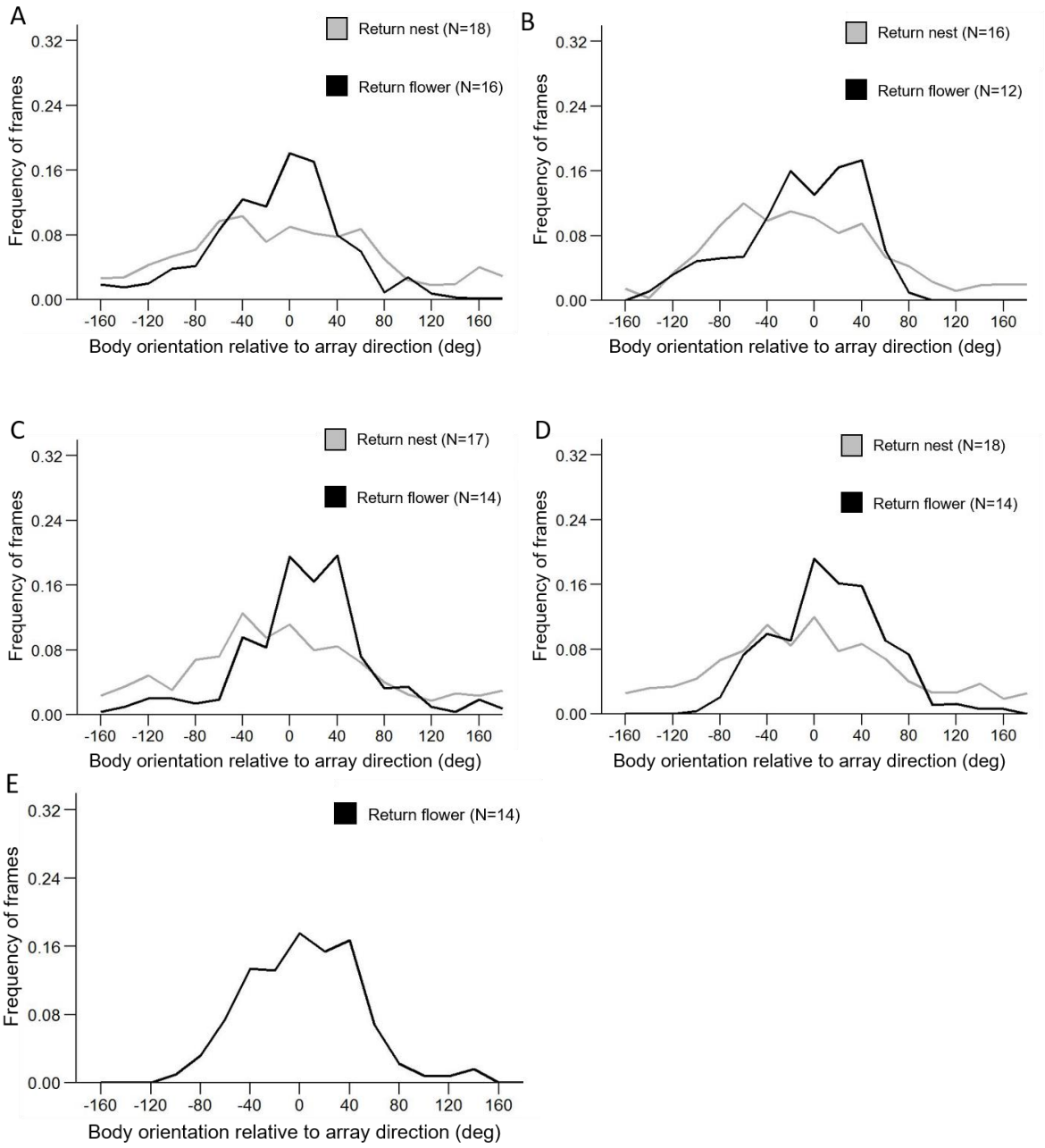


Figure 2.28. Frequency distribution of the bees' orientations relative to array-orientation for the first (A), second (B), third (C) and fourth (D) return flights to the nest (grey) or flower (black) location and the first approach of the flower prior to any experience (E). Approaches to flower: return 0: mean=2.22deg, rho=0.74; return 1: mean=-9.01deg, rho=0.64; return 2: mean=-5.81deg, rho=0.69; return 3: mean=13.89deg, rho=0.67; return 4: mean=12.06deg, rho=0.73. Approaches to nest: return 1: mean=-14.52deg, rho=0.33; return 2: mean=-20.11deg, rho=0.47; return 3: mean=-15.69deg, rho=0.38; return 4: mean=-12.09deg, rho=0.36. Bin width is 20°.

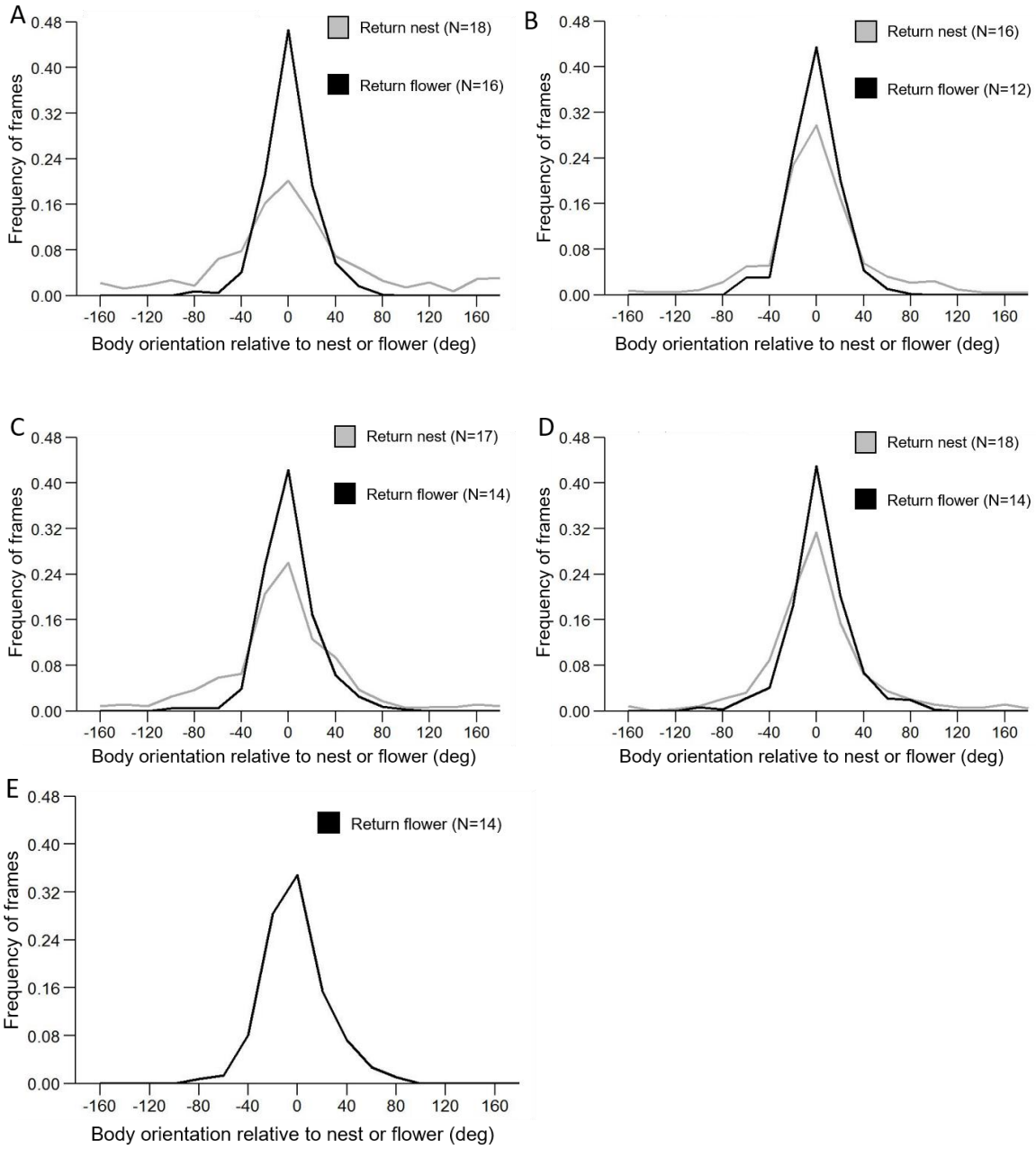


Figure 2.29. Frequency distribution of the bees' orientations relative to the goal for the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience (E). Approaches to flower: return 0: mean=1.95deg, rho=0.91; return 1: mean=-0.53deg, rho=0.94; return 2: mean=1.51deg, rho=0.94; return 3: mean=-0.08deg, rho=0.92; return 4: mean=-2.17deg, rho=0.90. Bin width is 20°.

When examining visually the figure 2.30, we can see that the distribution of the bees' body orientation during fixations relative to the flower, when binned according to compass direction, pointed roughly in the array direction as indicated by the presence of peaks around 0 degrees. However, for the three first returns to the nest, there are no clear peaks in the distribution. Such peak only appears in the distribution from the fourth return to the nest. Thus we can conclude that the fixations at the nest were distributed more broadly and were only directed toward the cylinders' array on the fourth return to the nest when the distribution of fixations resembled that of the fourth return to the flower (Watson-Wheeler test, W=0.72, p=0.70).

Most of the fixations relative to the flower and nest were directed at those goals (Figure 2.31). Fixations were strongly peaked towards the flower on all returns. Fixations were more scattered (Watson-Wheeler test, W=9.73, p=0.008) but still in the nest direction on returns to the nest. During the sequence of returns the distributions became more concentrated toward the nest and by the fourth return nest and flower distributions no longer differed (Watson-Wheeler test, W=4.33, p=0.11).

A visual examination of the figure 2.32 shows that most fixations of the flower and nest ($\pm 20^{o}$) occurred close to the goal as it is the case during learning flights (Figure 2.19). But in contrast to learning flights, the more scattered distribution of these fixation over the distance from the goals indicates that a substantial proportion of these fixations occur at longer distances from the flower and nest. In accord with more fixations at longer distances, a GEE model and a visual inspection of the figure 2.33 show that the rate of fixations is relatively constant across the flight, with no obvious increase close to the goal, in contrast to learning flights (Figure 2.20). Goal fixations during return flights may indicate when the bees are matching current views to those acquired closer to the goal during previous learning flights. For the first flight to the flower (RF00) to fit this suggestion, we must suppose that the bees

are matching views already encountered on the training table. As memories are labile when retrieved, bees may also acquire more information at these moments, or even make fixations specifically for acquiring new information on approaches (cf. Lehrer, 1993).

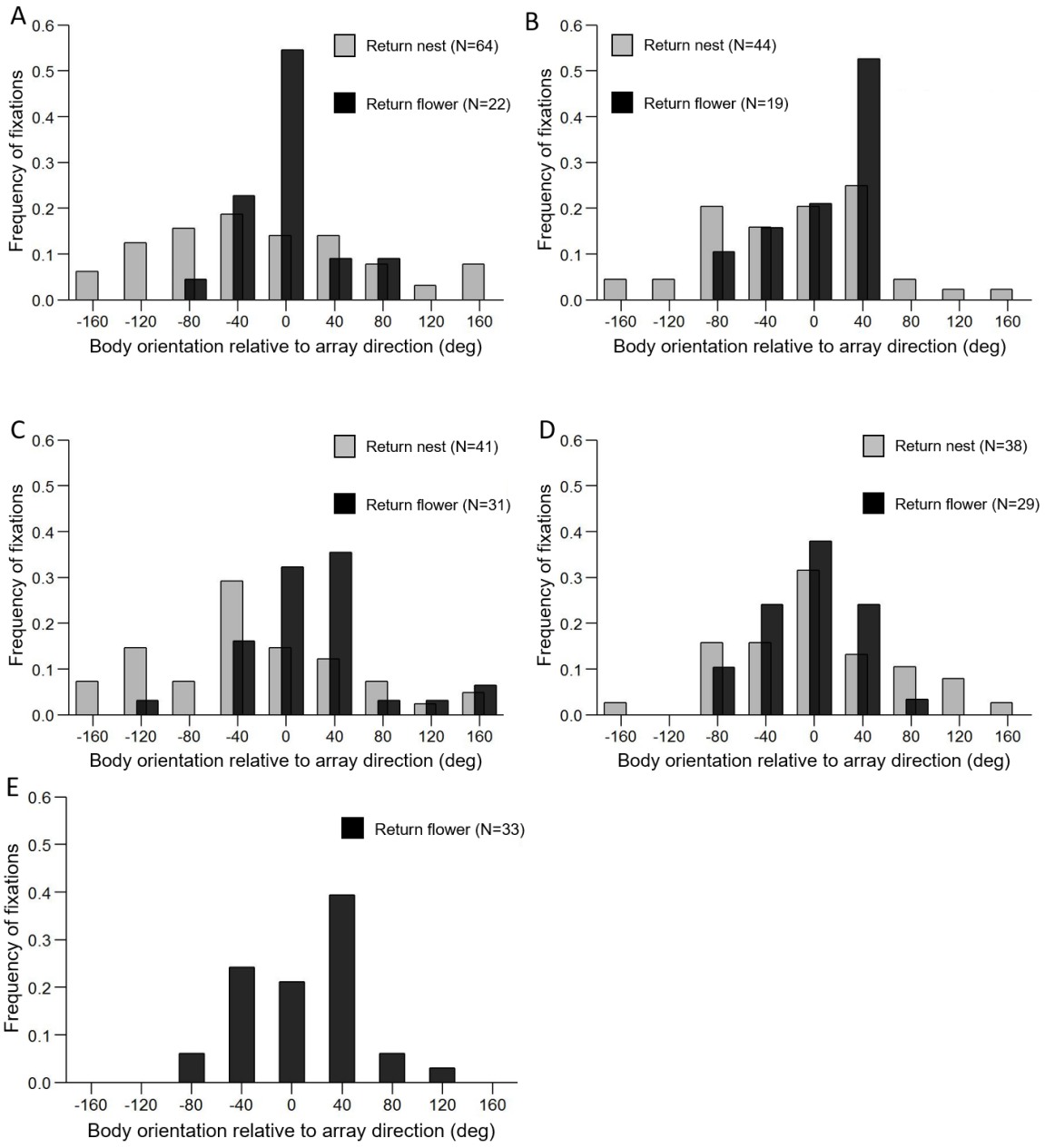


Figure 2.30. Frequency distribution of the bees' fixations relative to array-orientation during the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience of the location (E). Approaches to flower: return 0: mean=10.19deg, rho=0.74; return 1: mean=-4.01deg, rho=0.79; return 2: mean=4.82deg, rho=0.77; return 3: mean=17.72deg, rho=0.67; return 4: mean=-4.39deg, rho=0.77. Approaches to nest: return 1: mean=-40.88deg, rho=0.28; return2: mean=-14.99deg, rho=0.49; return 3: mean=-36.95deg, rho=0.36; return 4: mean=-0.44deg, rho=0.51 Bin width is 40°.

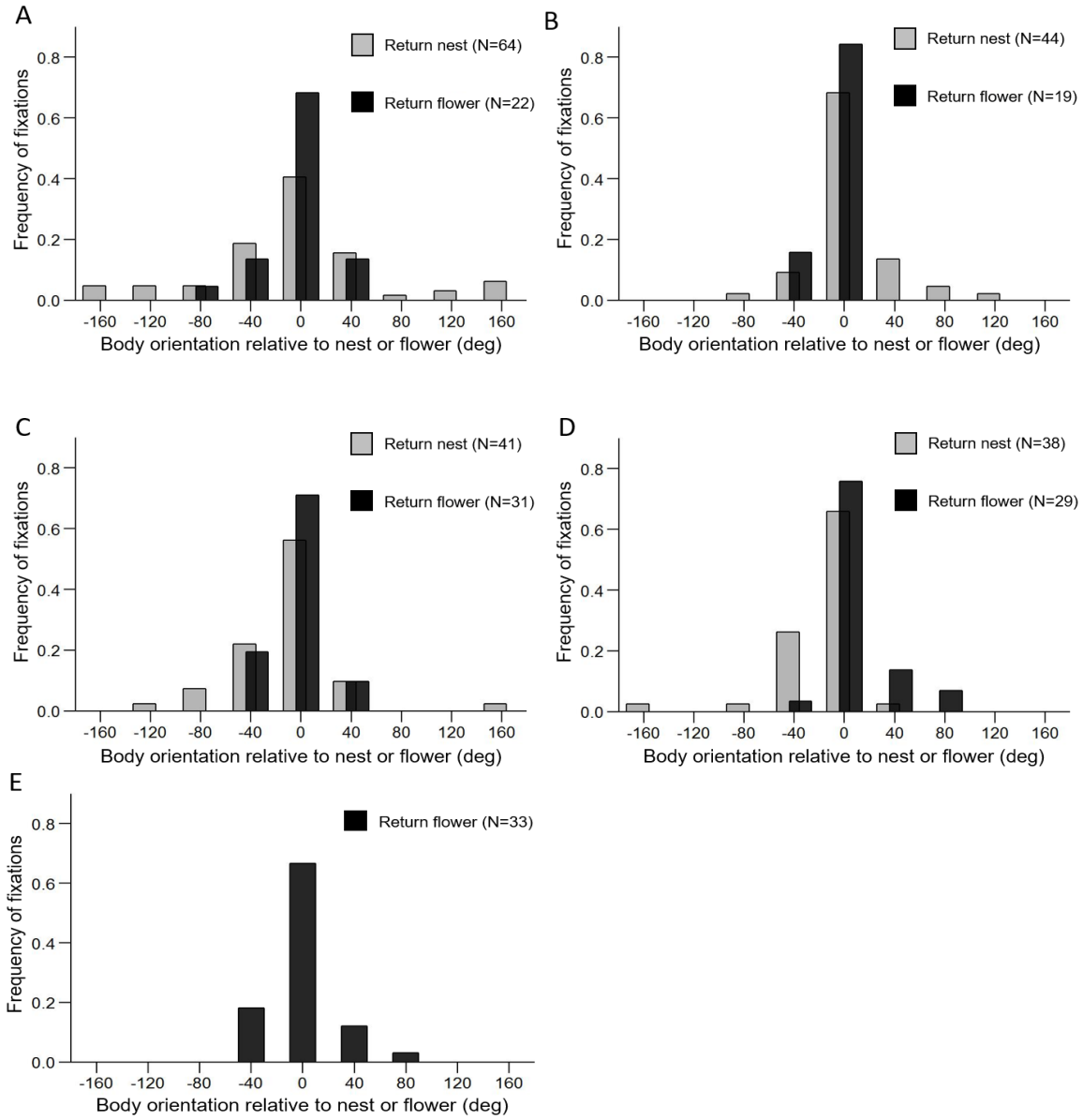


Figure 2.31. Frequency distribution of the bees' fixations relative to the goal during the first (A), second (B), third (C) and fourth (D) return flights at the nest (grey) or flower (black) location and the first approach of the flower prior to any experience of the location (E). Approaches to flower: return 0: mean=-3.46deg, rho=0.92; return 1: mean=-1.85deg, rho=0.92; return 2: mean=-8.10deg, rho=0.98; return 3: mean=-3.02deg, rho=0.94; return 4: mean=10.02deg, rho=0.92; Watson-Wheeler test across trial, W=12.97, p=0.11. Approaches to nest: return 1: mean=-6.23deg, rho=0.55; return 2: mean=1.68 deg, rho=0.87; return 3: mean=-12.19deg, rho=0.83; return 4: mean=-11.02deg, rho=0.89; Watson-Wheeler test across returns 1 to 4, W=19.1, p=0.004. Bin width is 40°.

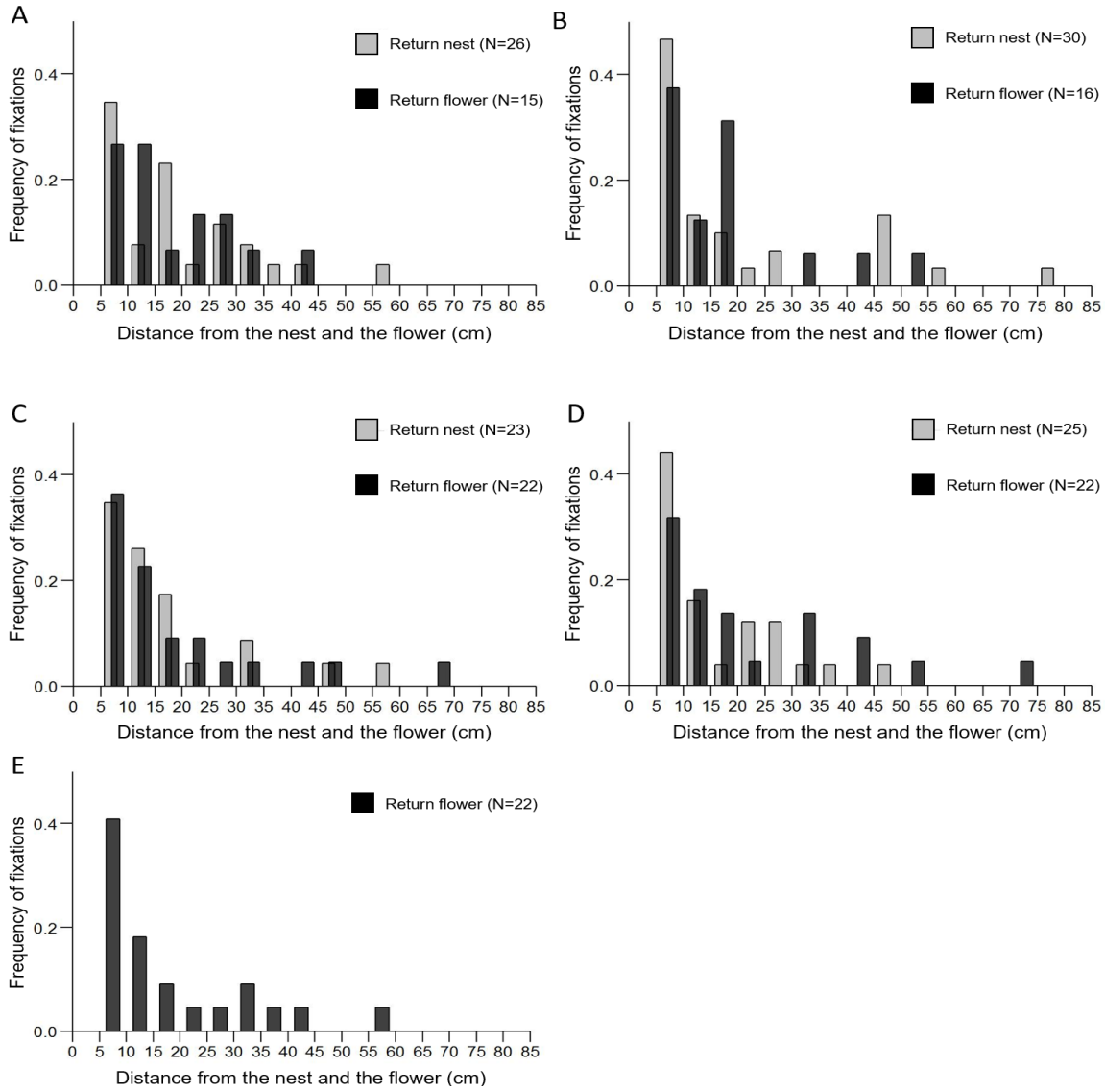


Figure 2.32. Normalised distribution of the facing fixations (±20°) depending on their distance from nest or flower during the first (A), second (B), third (C) and fourth (D) return flights and the first approach of the flower prior to any experience of the location (E). Mean fixation direction and rho during returns to the flower: return 0: mean=-3.46deg, rho=0.92; return 1: mean=-1.85deg, rho=0.92; trial 2: mean=-8.10deg, rho=0.98; return 3: mean=-3.02deg, rho=0.94; return 4: mean=10.02deg, rho=0.92; Watson-Wheeler test across trial, W=12.97, p=0.11. Mean fixation direction and rho during returns to the nest: return 1: mean=-6.23deg, rho=0.55; return 2: mean=1.68deg, rho=0.87; return 3: mean=-12.19deg, rho=0.83; return 4: mean=-11.02deg, rho=0.89; Watson-Wheeler test across returns 1 to 4, W=19.1, p=0.004. The bars show the proportion of the fixations oriented toward the nest (in grey) or the flower (in black) that fall into each 5cm distance bin.

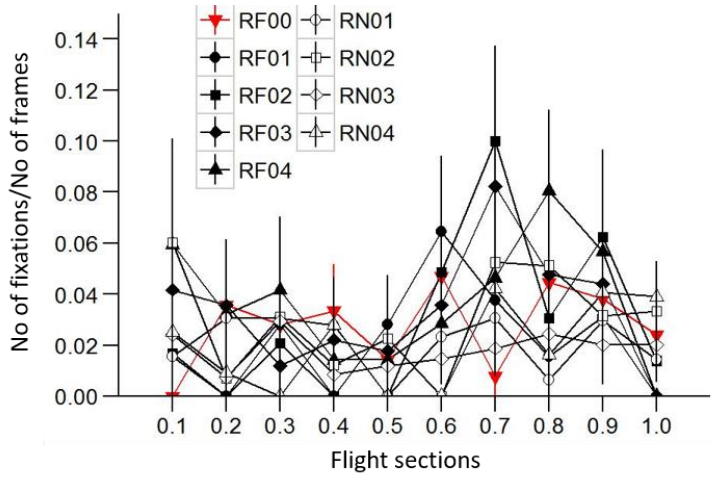


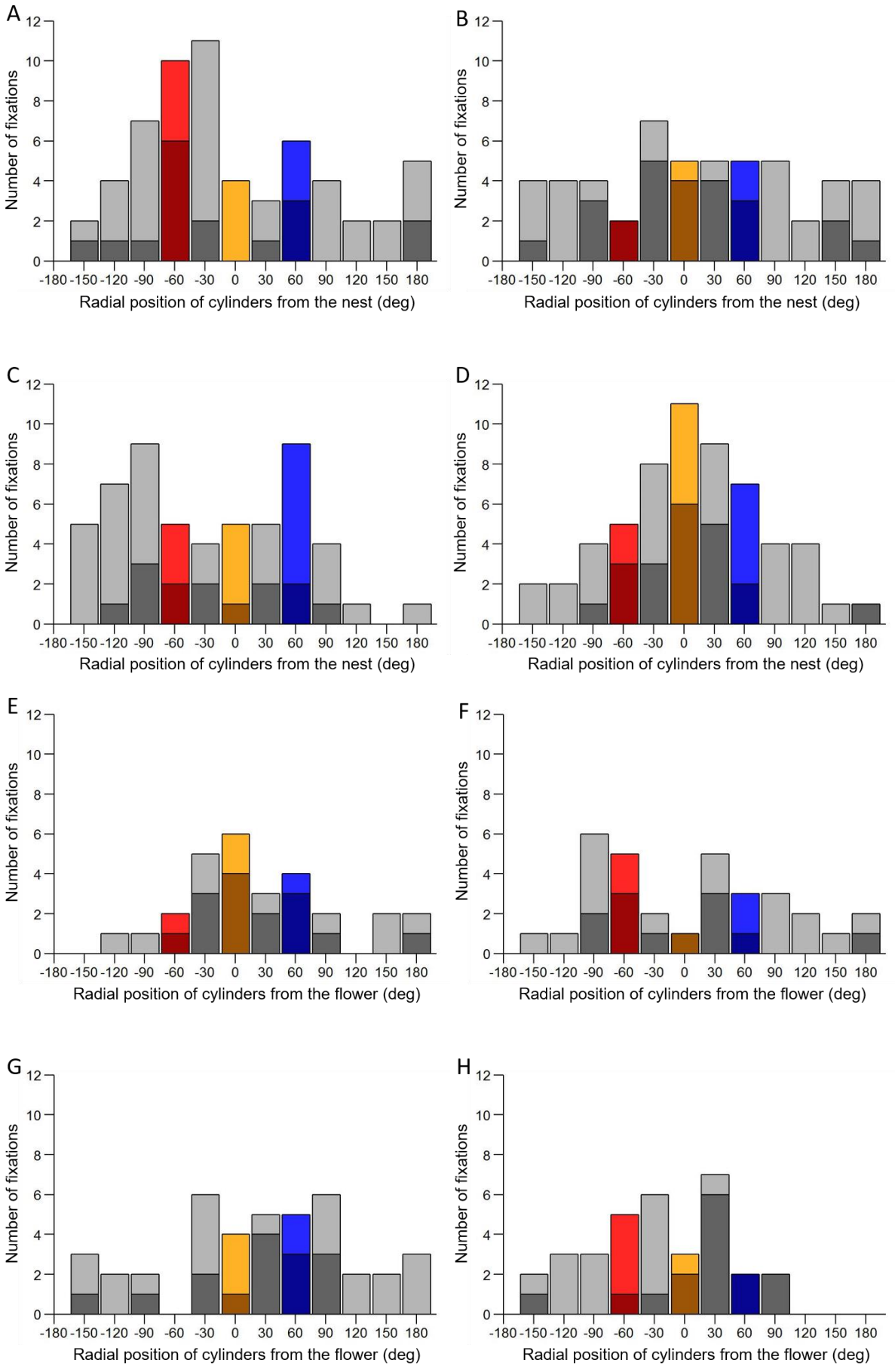
Figure 2.33. Mean rates of fixations centred on the goal (±20°) before to cross different distances from the nest (white symbols) or the flower (black symbols) during the return flights 1 to 4 and the first approach of the flower prior to any experience of the location (red symbols). For each bee, the number of fixations in each flight section is divided by the number of frames present in this flight section.

Table 2.4. GEE modelling examining the predictors influencing the bees' rates of fixation toward the goals (±20°) within each tenth of the return flights (number of fixations in the flight section/number of frames in the flight section). None of the predictors significantly influenced the fixation rates during the return flights.

Dependent Variables	Predictors	Estimates	Standard Error	$\chi 2$	*df*	*P*
Fixation rate	Flight section	1.06E-02	6.39E-03	2.78	1	0.096
	Trial	1.47E-03	2.15E-03	0.47	1	0.492
	Goal Nest	-7.81E-03	4.38E-03	3.18	1	0.075

2.3.8 Do the bees view the cylinders differently during returns to the nest and the flower?

To analyse cylinder fixations, I again compared the frequency of fixations towards real and virtual cylinders (cf. Figure 2.21), but a visual examination of the figure 2.34 does not reveal any clear difference in the number of fixations (Figure 2.34). In the figure 2.35, we can see that cylinder fixations, like nest and flower fixations, were less concentrated around the nest or the flower during returns than they were during learning flights. A sign that cylinder fixations are involved in guiding returns is that during the first return to the nest, the bees align the goal with the same cylinder that that most attracted their fixations during their first learning flight (Figure 2.35). Similarly, bees on their first learning flight from the flower and their first return to the flower fixated the northern cylinder. Thus, in early returns joint fixations of cylinder and goal may help bees reach the nest and the flower (cf. Zeil 1999; Philippides et al. 2013).


A
B
C
D
E
F
G
H
Number of fixations
0
2
4
6
8
10
12
-180 -150 -120 -90 -60 -30 0 30 60 90 120 150 180
Radial position of cylinders from the nest (deg)
Radial position of cylinders from the flower (deg)

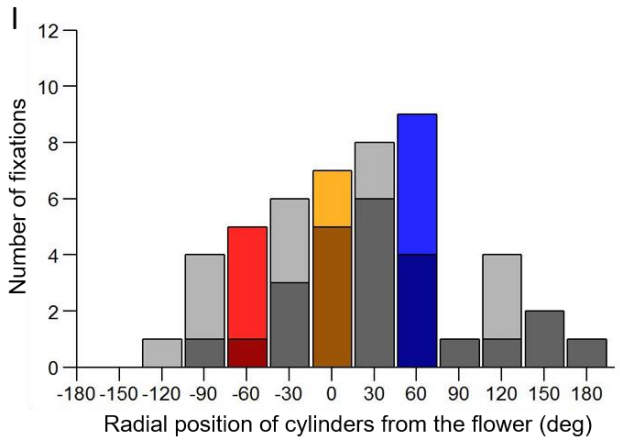
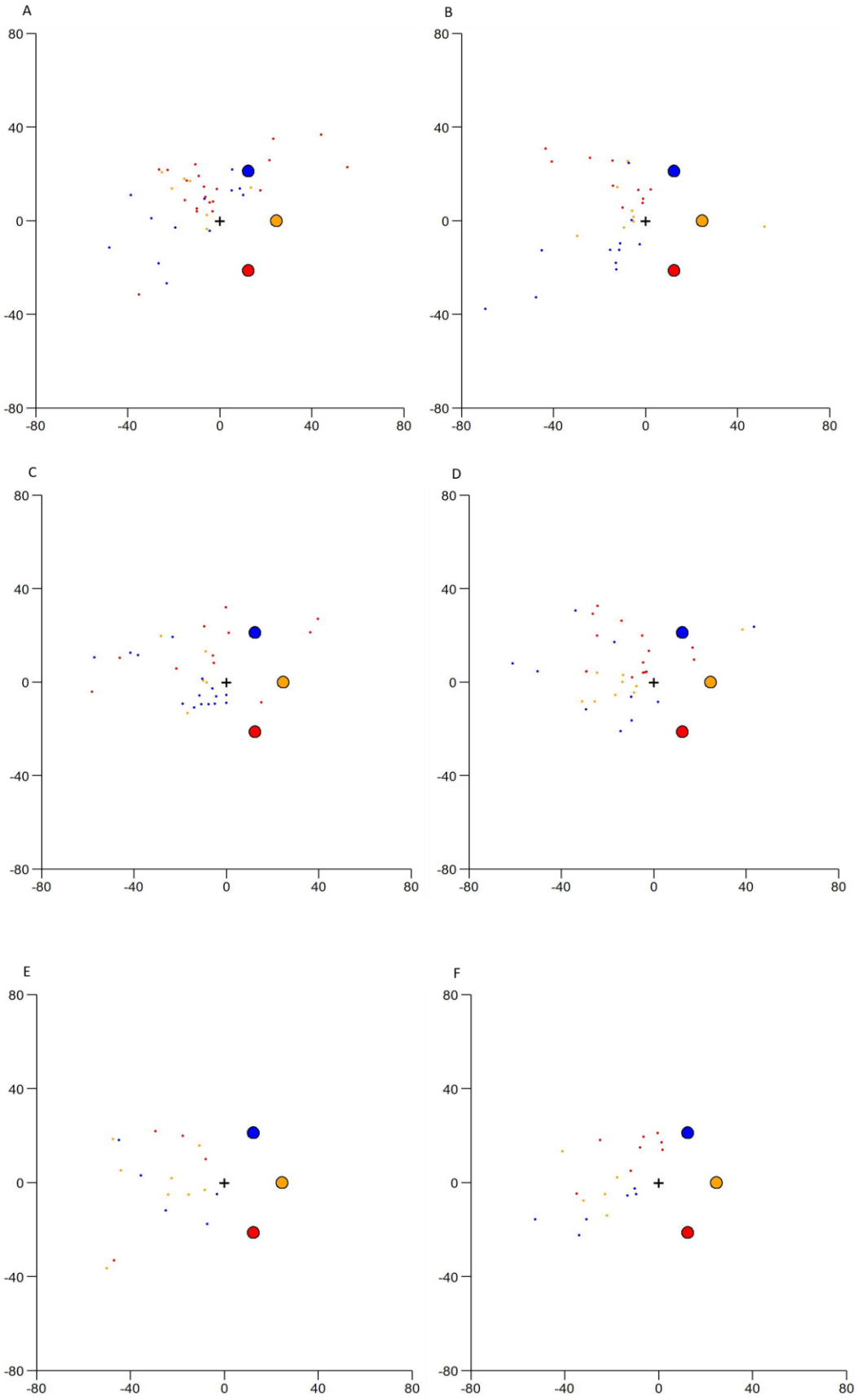


Figure 2.34. Number of fixations toward the real (coloured) and virtual (grey) cylinders (±10°) depending on their radial position around the nest during the first (A), second (B), third (C) and fourth return flights (D) or around the flower during the first (E), second (F), third (G) and fourth return flights (H) and the first approach of the flower prior to any experience of the location (I). The darker bars indicate the number of the fixations that are also toward the nest or the flower (±10°).


A
B
C
D
E
F
80
40
0
-40
-80
-80
-40
0
40
80

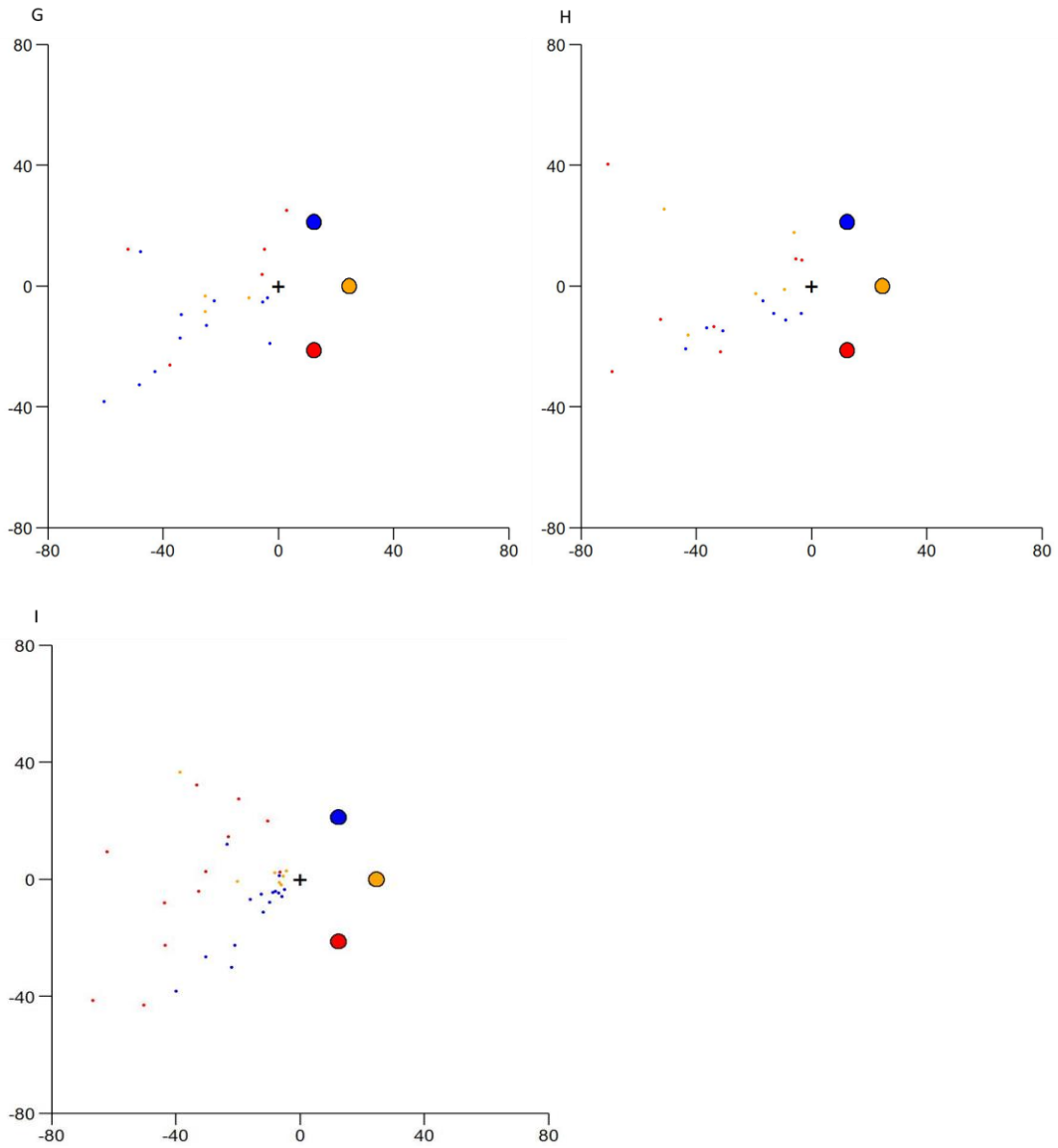


Figure 2.35. Bees' position during fixations toward the cylinders (±20°) during the first (A), second (B), third (C) and fourth (D) return flights at the nest and the first (E), second (F), third (G) and fourth (H) return flights at the flower location and the first approach of the flower prior to any experience of the location (I). Each of the points represent the position of a bee during the first frame of a fixation toward the left (in blue), central (in yellow), or right cylinder (in red). The corresponding cylinders' positions are shown by the large coloured circles. The nest or flower location is marked by a black cross.

2.3.9 Tests

The details of both learning and returns flights suggest that bees learn different features of the visual scene containing their nest and a flower. Tests in which the scene is altered are needed to examine what these differences might be. So far only two tests have been conducted and analysed.

In the tests, the array of cylinders was shifted relative to its normal position. In one test at the feeder and in one test at the nest the ring was shifted with the array of cylinders. In a second pair of tests the ring was removed and bees could only use the cylinders to pinpoint the goal. The bees' knowledge of the location of the nest and flower was examined in two ways. The most straightforward was to score their landing positions. Since the bees slowed down during normal returns as they were near the goal, a second possible measure is to examine where during their approach they slowed down.

After a visual analysis of the figure 2.36, the results were surprising. Despite the longer and more elaborate learning flights at the nest, bees were equally accurate in their landings at both locations. They approached the two goals and landed at precisely the right location relative to the cylinders when the rings were present and over a larger area when the rings were removed. A GEE model shows that the distributions of distances from the virtual nest with no ring (median 6.75cm, IQR=6.47) was similar to the distances from the virtual flower site with no ring (median 5.8cm, IQR=4.23), (Figure 37 A and B; table 2.5.1). After each approach and landing, the bees tended to repeat their landings before they flew away again and performed another approach (Figure 2.36). A second GEE model confirmed that the distribution of distances from the goal of these repeated landings were very similar to the first landings at virtual nest and flower positions (nest site no ring: median 6.07cm, IQR=5.89; flower site no ring: median 5.22cm, IQR=5.10; Figure 2.37 C and D ; table 2.5.2).

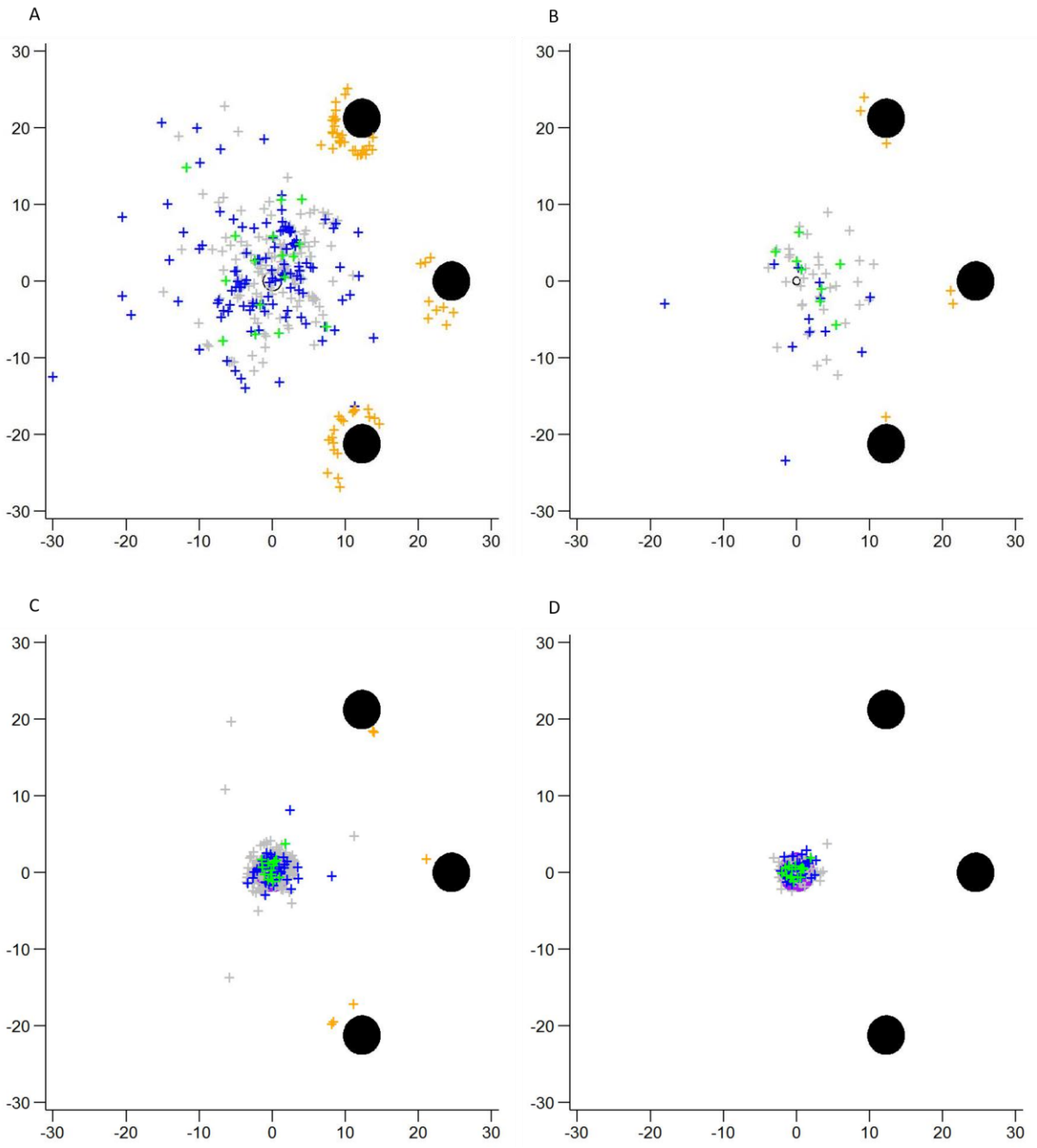


Figure 2.36. Positions of the first landing (green crosses), landings after approaches (blue crosses), repeated landings (grey crosses) and at the cylinders (orange crosses). The virtual position of the goals relative to the cylinders are marked by empty black circles for the tests without purple ring at the nest (A) and the flower location (B). The same goals locations are shown by purple circles for the tests with the rings placed on the nest (C) and flower (D) tables. The cylinders' positions are shown by the large black filled circles.

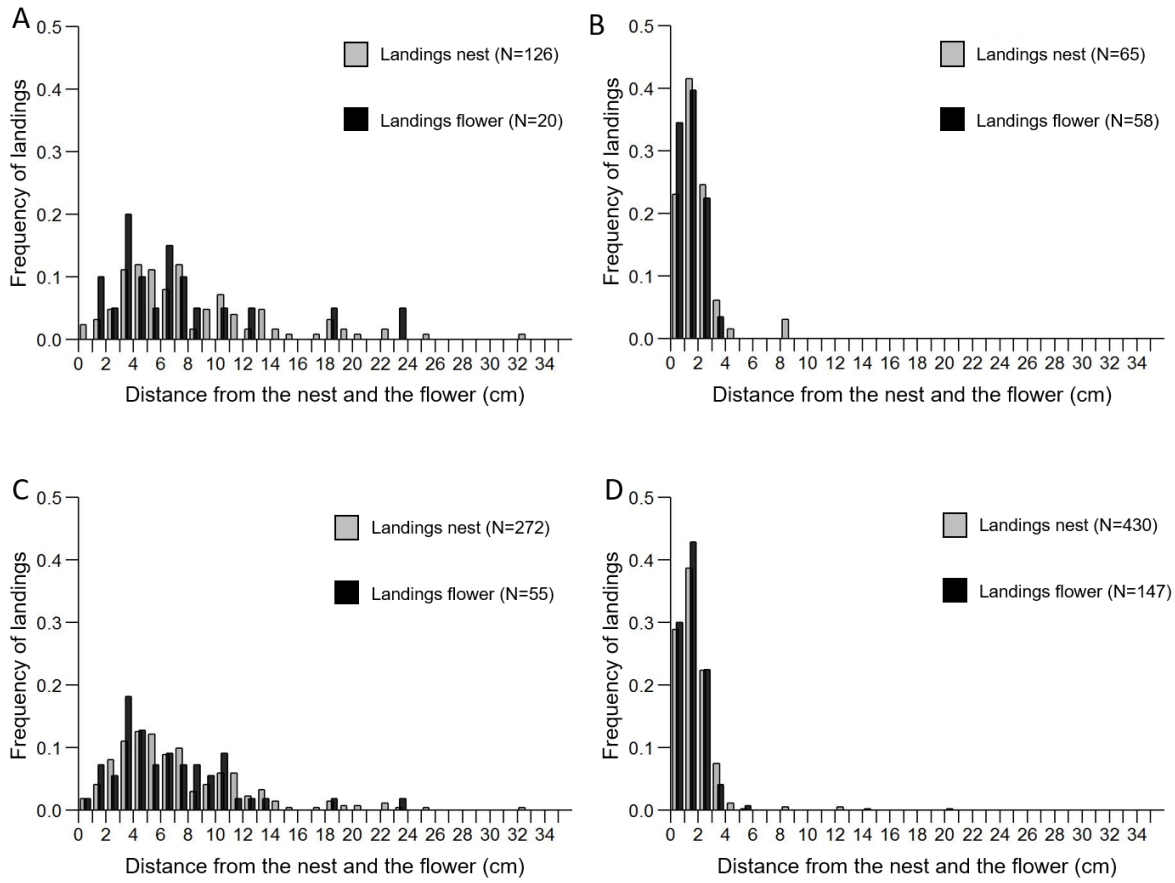


Figure 2.37. Normalised distributions of the landings following approaches depending on their distance from the virtual position of the goals computed relative to the position of the cylinders for the tests without rings (A) and with rings (B) at the nest (grey) and flower location (Black). The same plots including the repeated landing are shown for the test without the rings (C) and with the rings (D).

In both nest and flower tests, when the ring was removed, the bees sometimes landed at the foot of one of the cylinders and stayed there for a short time before flying again (Figure 2.36). It seems unlikely that bees mistook the cylinders for the goal, but they may well know that the cylinders are close to the goal and so a good place to rest when the absence of the ring increases the bees' uncertainty of the location of the goal.

When the ring was present, so that the visual scene was normal, the bees were more certain that the cylinders and ring did mark the site of the nest or flower (Figure 2.36). In these conditions, the GEE model shows that the landings were almost all very close to the centre of the ring (distance from centre of ring at nest: median 1.57cm, IQR=1.16; at flower median 1.23cm, IQR=1.05. Repeated landings at the nest: median 1.49cm, IQR=1.35; and the flower: median 1.33cm, IQR=1.13; Figure 2.37, Table 2.5).

The visual analyses of the figures 2.38, 2.39 and 2.40 shows that the positions in which bees slowed down without landing in tests with no ring were nearly as accurate as when the bees' landed (Figure 2.38). In terms of precision there was little difference between the bees' distance from the nest (median 10.20cm, IQR=10.20) and flower (median 7.71cm, IQR=6.51) (Figure 2.39 A). But the spatial organisation of these slow speed positions did differ between nest and flower tests. Slowdowns around the virtual nest were distributed along an axis perpendicular to the array direction and at the flower they spread along an axis parallel to the array direction. Slowdowns at both sites were biased to be further from the cylinders than the virtual goal (Figure 2.40), suggesting that the bees estimated their distance from the cylinders and avoided too close an approach. It is clear that bees were guided by the cylinders during both nest and flower tests.

In tests with rings, the bees mostly slowed down close to the ring, but there were also outlying points at some distance from the ring. A Gee model indicates that the distances

were thus similar between the nest (median 4.18cm, IQR=11.90) and flower (median 2.93cm, IQR=3.15) and also between tests with and without rings (table 2.5.3).

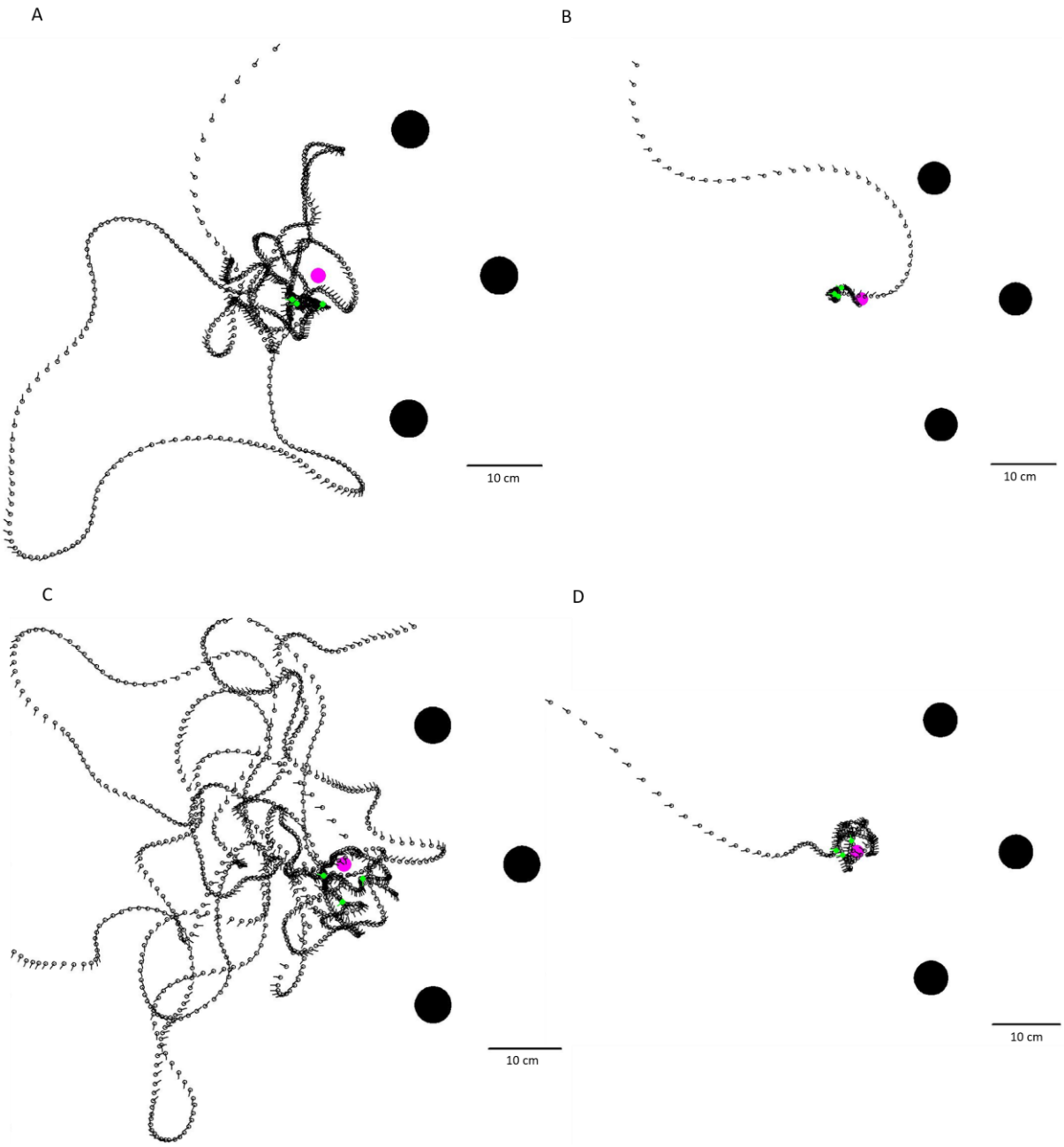


Figure 2.38. Examples of trajectories during the early search bouts of the tests at the nest location without (A) and with (B) purple ring and at the flower locations without (C) and with the rings (D). The large black filled circles represent the position of the cylinders. The purple circles show the virtual locations of the nest or the flower computed relative to the position of the cylinders. The empty circles are the positions of the bee plotted for frames and the tails represent the orientation of the bee. In green are the three slow down locations detected by our code.

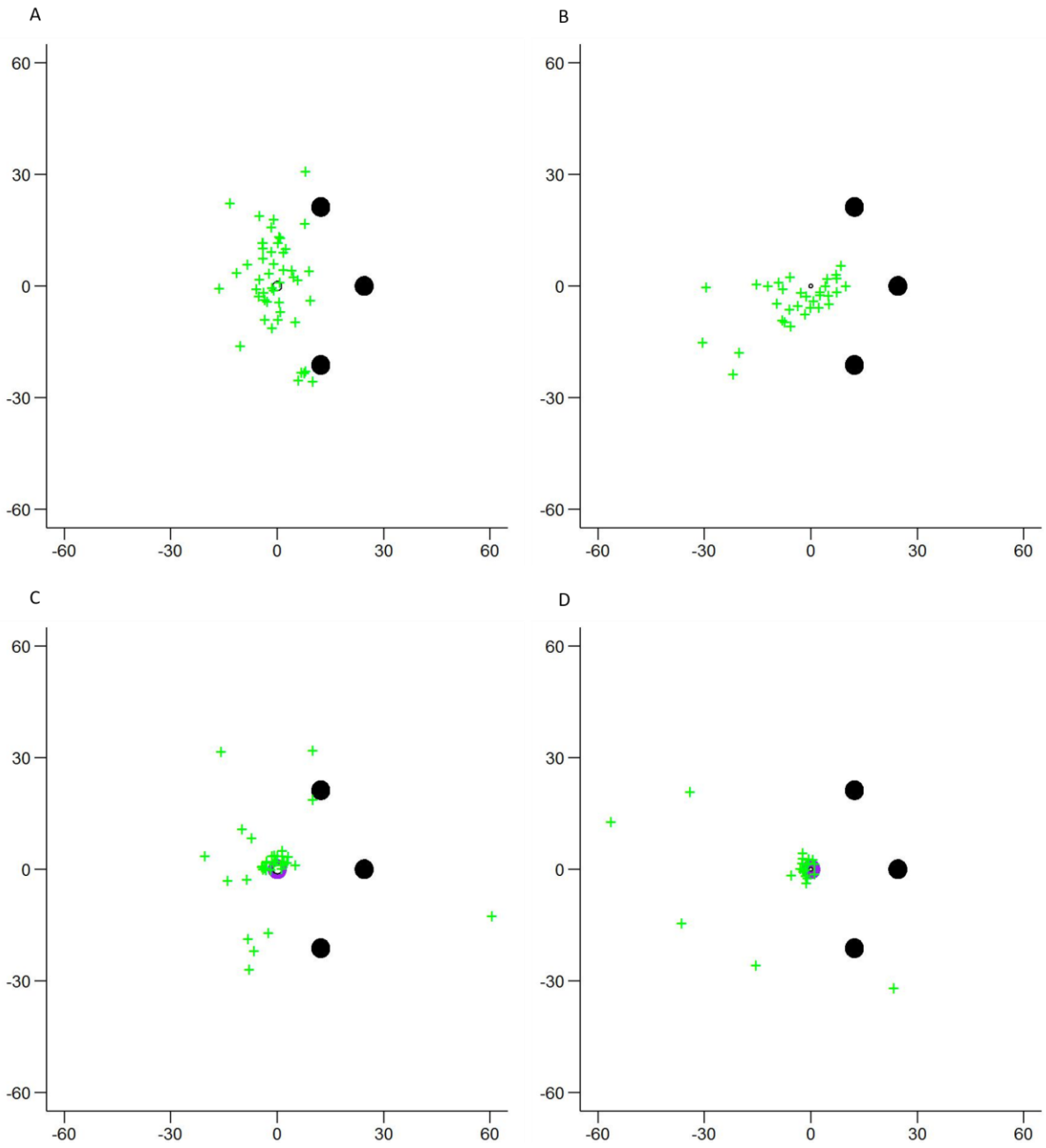


Figure 2.39. Positions of the slow down points (green crosses) during the early search bouts. The virtual position of the goals relative to the cylinders are marked by empty black circles for the tests without purple ring at the nest (A) and the flower location (B). The same goals locations are shown by purple circles for the tests with the rings placed on the nest (C) and flower (D) tables. The cylinders' positions are shown by the black filled circles.

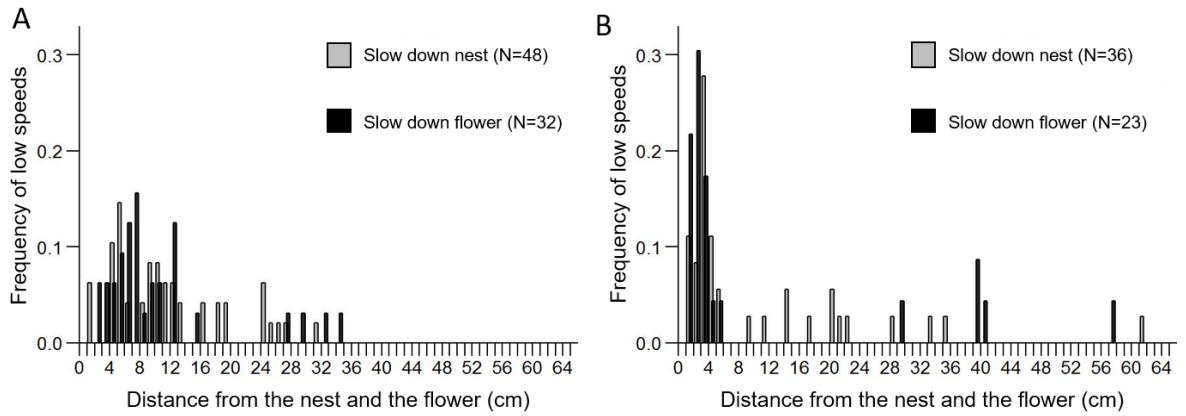


Figure 2.40. Normalised distributions of the slow down points depending their distance from the virtual position of the goals computed relative to the position of the cylinders for the tests without rings (A) and with rings (B) at the nest (grey) and flower location (Black).

The slowdowns are useful as we can see where the bees looked when they are relatively close to the goal. We examined the bees' body orientation relative to compass direction, the nest and flower and to each cylinder when the ring was present and when it was missing. The data with the ring is more informative, probably because the bees were relatively more certain of their location and very close to the virtual goal. With the ring present, visual inspection of the figure 2.41B shows that the bees faced both the centre of the flower and the nest hole. The visual analysis of the distributions of compass orientation presented in Figure 2.42B indicates that the distribution resembled closely the distributions of compass directions during the first phase of the first learning flights from the nest and flower (Figure 2.13). Consequently, the cylinders are also viewed roughly as they are during the first phase of the learning flight with bees facing the central cylinder with the right cylinder to the east and the left cylinder to the west, as shown a visual examination of the Figures 43B, 44B and 45B. Similarly, in nest searches with the ring, bees tended to fixate the central cylinder most strongly, as also happened on the first learning flight (Figure 2.20) and on the 4th return, the eastern cylinder was fixated a little less strongly than in the first learning flight and the left cylinder has a peak

to the east. Possibly the weaker peaks in the nest searches is because bees have a greater tendency to look around at the visual scene when finding their nest hole than do bees looking for a flower.

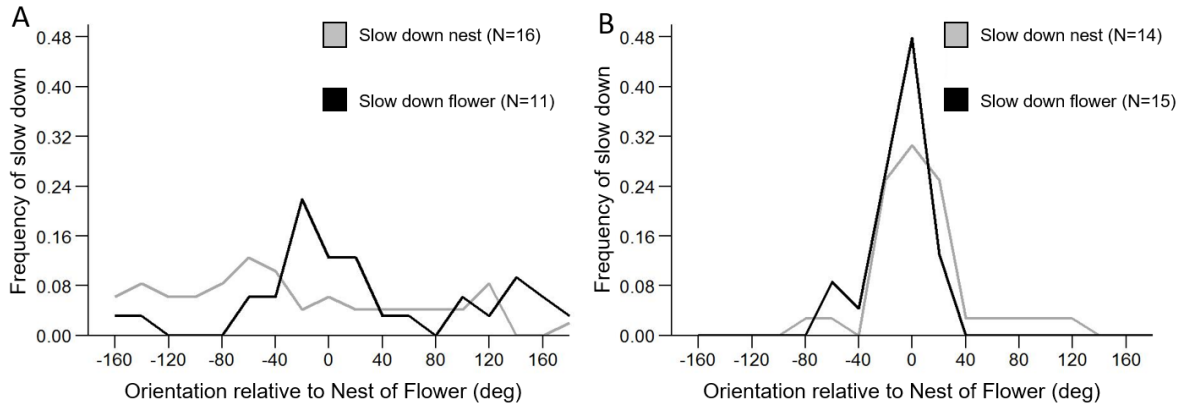


Figure 2.41. Frequency distribution of the bees' orientations relative to the virtual position of the nest (in grey) or the flower (in black) during their slow down points for the tests without (A) and with (B) the rings on the experimental tables. Bin width is 20°.

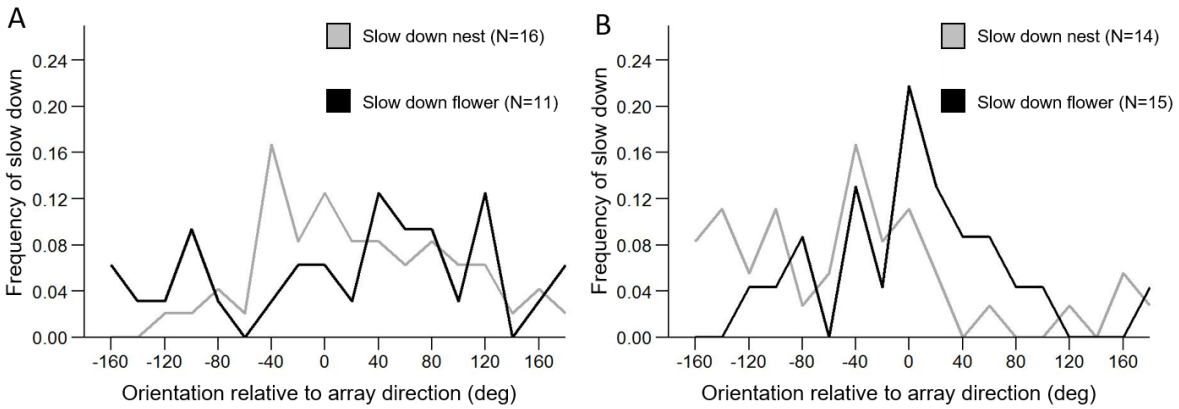


Figure 2.42. Frequency distribution of the bees' orientations relative to array direction during their slow down points for the tests without (A) and with (B) the rings on the experimental tables at the nest (in grey) and flower (in black) locations. Bin width is 20°.

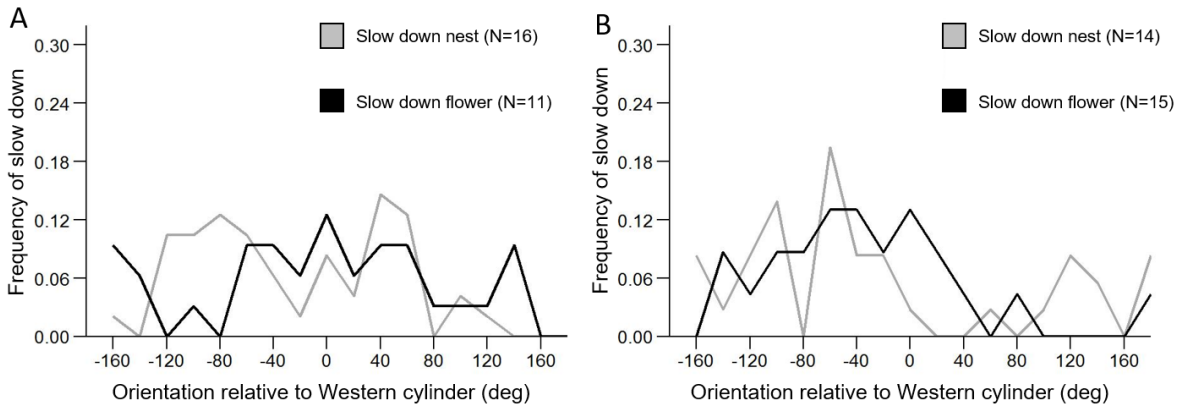


Figure 2.43. Frequency distribution of the bees' orientations relative to the left cylinder during their slow down points for the tests without (A) and with (B) the rings on the experimental tables at the nest (in grey) and flower (in black) locations. Bin width is 20°.

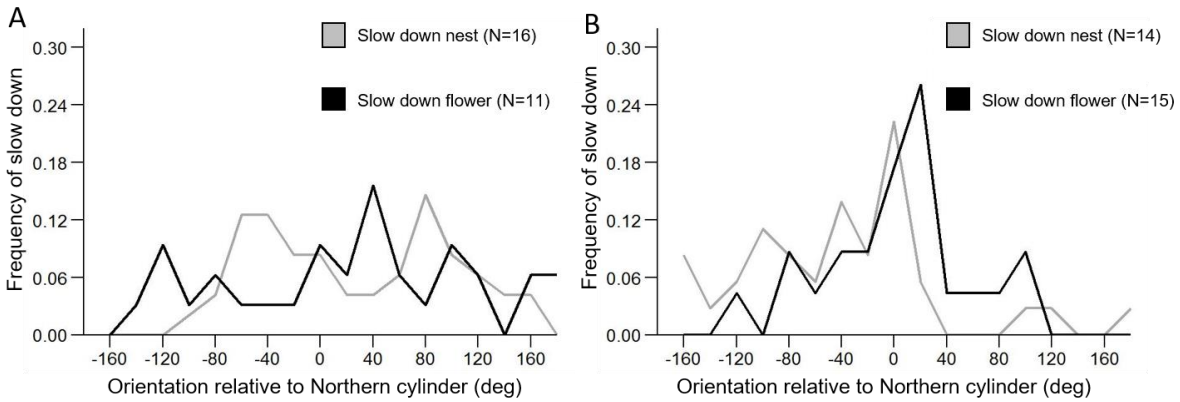


Figure 2.44. Frequency distribution of the bees' orientations relative to the central cylinder during their slow down points for the tests without (A) and with (B) the rings on the experimental tables at the nest (in grey) and flower (in black) locations. Bin width is 20°.

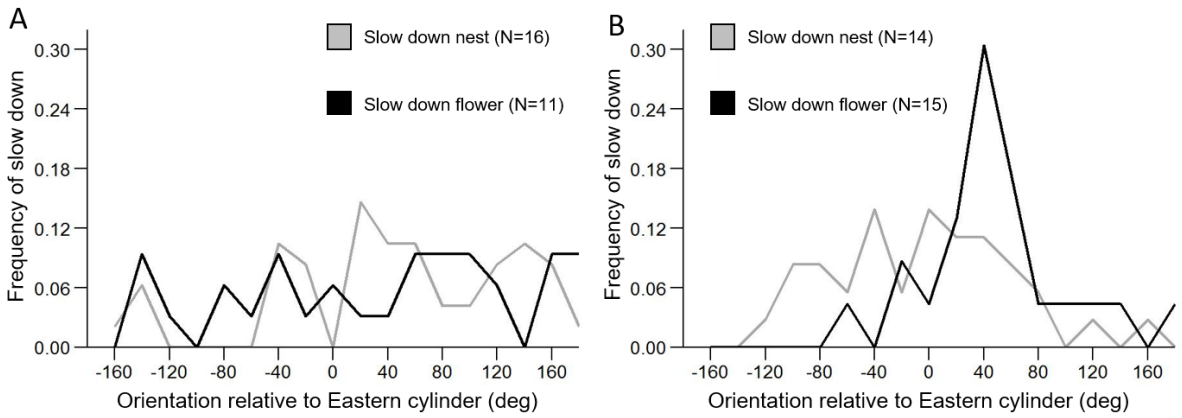


Figure 2.45. Frequency distribution of the bees' orientations relative to the right cylinder during their slow down points for the tests without (A) and with (B) the rings on the experimental tables at the nest (in grey) and flower (in black) locations. Bin width is 20°.

When visually analysing the figures 2.41A and 2.42A, we can see that the distributions of viewing directions were much more uniform when the ring was missing. The only convincing peaks are the compass direction of the bees' body orientation during nest searches (Figure 2.42A) and their body orientation relative to the flower during flower searches (Figure 2.41A). The bees' peak compass orientation during nest searches without the ring was about the same as it was in the presence of the ring. But the peak orientation relative to the flower shifted about 20^{o} to the west. A possible but speculative interpretation of these results is that bees have both a preferred compass body orientation and a preferred viewing body orientation within the scene. These preferences will match when the bee is close to the goal but can diverge when the bee is further away. When they do diverge, bees adopt a weighted compromise orientation. In the flower searches bees are located predominantly to the east of the array so a compromise orientation direction would shift their orientation to the west. The nest search positions are distributed equally about the nest-array axis so that some bees would shift their orientation east and others west resulting in a symmetrical distribution. A simpler alternative suggestion is that the bees' orientation relative to the flower is the outcome of

positional view matching within the scene and that view matching during the widely distributed slowdowns in nest searches produces more diffuse orientations.

Table 2.5. GEE modelling examining the effect of the presence or absence of the purple ring and the location on several indicators of the bees' search accuracy during the tests. Entry 1: The landings following approaches were more distant from the virtual positions of the goal when the ring was remove than when it was left on the table. They did not vary between locations. Entry 2: The landings following approaches and repeated were more distant from the virtual positions of the goal when the ring was remove than when it was left on the table. They did not vary between locations. Entry 3: The distances of the slowdowns from the virtual positions of the goal did not vary between the two locations and whether the ring was on the table or not.

	Dependent Variables	Predictors	Estimates	Standard Error	$\chi 2$	*df*	*P*
1	Distance of landings	Ring removed	6.3	0.52	149.21	1	**<0.001**
		Goal Nest	0.69	0.39	3.06	1	0.08
2	Distance of landings	Ring removed	5.41	0.39	192	1	**<0.001**
		Goal Nest	0.45	0.28	2.67	1	0.1
3	Distance slow speeds	Ring removed	0.105	2.522	0.015	1	0.9
		Goal Nest	0.223	1.837	0.192	1	0.66

2.3.10 Are the bees more persistent at the nest than at the flower?

Another way that bees learning about the location of a flower may differ from their learning of the location of their nest is in the strength of the memory as shown by the longer and more elaborate learning flights at the nest than at the flower. Longer learning and better memories

may well be evident in the persistence of the bees' search at the two locations: a nest is forever, but a flower may only be a one day's stand. A further interesting question is how the persistence of the bees' search may be modulated by the presence or absence of the ring. Searching for a flower which has disappeared from its former location makes less sense than searching for a nest that lacks one of several cues marking its location.

To analyse the bees' persistence, I computed 3 measures for each bee: I) the number of landings after approaches per second, II) the number of landings including the repeated ones per seconds, III) the mean duration of the search bouts.

A Gee model shows that the measure I), the rate of landings following approaches, was influenced by both the location, nest or flower, and by the whether the ring was there or not (table 2.6). When the ring was absent the rate of landings was higher at the nest (median 0.04landings/s, IQR=0.02) than at the flower (median 0.00landings/s, IQR=0.04) (Wilcoxon test, N=17, W=20, Z=-2.57, p=0.01). But when the ring was present the rate of landings was unchanged at the nest (median 0.04 landings/s, IQR=0.01) but was much greater during flower searches (median 0.08 landings/s, IQR=0.02) making persistence at the flower greater than at the nest (Wilcoxon test, N=16, W=100, Z=-3.41, p<0.001).

A second GEE model demonstrates that the measure II), the rate of landings following approaches and repeated landings, gave somewhat similar results (table 2.6). In the absence of the ring the rate of landings was greater during nest searches (0.08 landings/s±0.05) than during flower searches (0.00 landings/s, IQR=0.07) (Wilcoxon test, N=17, W=30, Z=-2.11, p=0.03). The presence of the ring increased the rate of landings above that when there was no ring that (table 10.2) in both nest (Wilcoxon test, N=16, W=4, Z=-3.31, p<0.001) and flower searches (Wilcoxon test, N=16, W=0, Z=-3.52, p<0.001) such that the rate of landings did not differ between nest (median 0.24 landings/s, IQR=0.10) and flower searches (median 0.22 landings/s, IQR=0.10) (Wilcoxon test, N=16, W=30, Z=-1.45, p=0.1).

Finally, a last GEE model shows that the measure III), the mean duration of the search bouts gave results that differed from Measures I and II (table 2.6). In the absence of the ring nest searches were longer (median 14.70s, IQR=7.82) than flower searches (median 5.58s, IQR=6.47) (Wilcoxon test, N=17, W=4, Z=-3.43, $p<0.001$). When the ring was present nest searches continued to be longer (median 31.50s, IQR=23.10) than flower searches (median 7.95s, IQR=3.16) (Wilcoxon test, N=16, W=0, Z=-3.52, $p<0.001$). Contrary to measures I and II, nest searches were significantly longer with the ring (Wilcoxon test, N=16, W=5, Z=-3.26, p=0.001). But the ring had no significant effect on the length of flower searches (Wilcoxon test, N=16, W=30, Z=-1.86, p=0.06).

Taken together, these results indicate that bees search more persistently for the nest than for the flower and that the presence of the ring can, but does not always, make bees search for longer. But, as explained in the Discussion, these results do not prove that nest memories are stronger than flower memories.

Table 2.6. GEE modelling examining the effect of the presence or absence of the purple ring and the location on several indicators of the bees' search persistence during the tests. Entry 1: The rate of landings following approaches was higher when the ring was present than when it was removed and was higher at the nest location than at the flower location, but the two effects were also in interaction. Entry 2: The rate of landings following approaches and repeated was higher when the ring was present than when it was removed and was higher at the nest location than at the flower location. Entry 3: The mean search bouts were longer when the ring was present than when it was removed and were longer at the nest location than at the flower location, but the two effects were, here again, in interaction.

	Dependent Variables	Predictors	Estimates	Standard Error	$\chi 2$	*df*	*P*
1	Landing rate	Ring present	0.075	0.011	34.4	1	**<0.001**
		Goal Nest	0.025	0.007	35.2	2	**<0.001**
		Interaction	-0.075	0.013	47.1	2	**<0.001**
2	Landings rate	Ring present	0.165	0.017	94.6	1	**<0.001**
		Goal Nest	0.04	0.017	5.67	1	**0.017**
3	Mean bout duration	Ring present	1.46	1.19	11.8	2	**0.003**
		Goal Nest	10.13	2.06	42.1	2	**<0.001**
		Interaction	28.2	9.31	9.17	1	**0.003**

2.4 Discussion

The major difference between learning flights from the nest and flower is that the flights from the nest are much longer, with trajectories about twice the length of those from the flower. Both at the nest and flower the learning flights tend to become shorter with each departure, but this happens more rapidly with flights from the flower than the nest. During their longer trajectories, bees leaving the nest survey the immediate nest surroundings over a larger area than they do the flower. During this survey, bees make brief fixations of the nest, the flower

and the three cylinders marking these locations. These fixations could potentially be when bees acquire views of their surroundings and differ between flights from the flower and the nest.

The rate and number of fixations is higher in flights from the nest than from the flower and fixations also occur over a longer range of distances from the goal. There are also differences in the pattern of fixations during nest and flower flights. On the first learning flight from the nest and also on the first return flight to it, bees tend to fixate the two peripheral cylinders of the array. In contrast, on the first flight from and on their return to the flower, the bees tend to fixate the central cylinder. The timing of cylinder fixations also differs between learning flights from the nest and the flower. When leaving the nest, the peak rate of cylinder fixations happens right at the start of the flight, as do nest fixations. On departures from the flower, the peak rate of both flower and cylinder fixations is delayed until about a third of the way through the flight. The peak fixation rates are similar for nest and flower, but the fixation rates of cylinders are lower than in flights from the nest.

Taken together, these findings suggest that the bees' memories of their nest and its setting are stronger and that the bees acquire views from more vantage points and in more directions than they do during flights from the flower. In particular, the different rates of cylinder fixations between nest and flower learning flights suggest that bees place greater significance on learning about the cylinders in nest flights. In what follows these findings are discussed first from an ecological perspective and second whether bees in these experiments do indeed demonstrate that their memories of the nest are stronger than their memories of the flower.

There are obvious ecological reasons why bees might invest more in learning about their nest than a flower and why what they learn there may differ at the two locations. The nest hole of many insects, including *Bombus terrestris*, is inconspicuous and learning its location may be hard. Many flowers, on the other hand, are designed to be conspicuous and easy to spot.

Detailed knowledge of a flower's location may not even be required. Patch location can be sufficient as individual flowers are often set among others of the same species and an individual flower is often transient, certainly in comparison with the bees' nest hole. It is likely to be equally important for bees to learn to recognise the flower, so that they can find the same flower elsewhere.

There are interesting differences in the learning flights of honeybees and bumblebees leaving flowers which correlate well with the notion that flight length is related to memory strength. Bumblebee flights are relatively short compared with those of honeybees. Wei et al. (2002) report a mean duration of 15s in honeybee flying in cluttered surroundings with the longest flight being 26s and Lehrer (1993) also cites durations that are longer than the bumblebees in the current experiments. She also found that learning flights in honeybees continue over 6 departures rather than 2 to 3 in bumblebees. It is hard to attribute these differences to the surroundings or the rewards at the flowers. The differences correlate with the bees' foraging behaviour and may well be a property of the species.

Several studies have shown that bumblebees are less flower constant than honeybees (Free, 1963, 1970; Gegear & Laverty, 2004). Bumblebees are also faster than honeybees to discover and switch to a new food source if the quality of food source drops (Townsend-Mehler, Dyer, & Maida, 2011). Bumblebees like to forage on several species at once (Heinrich, 1979) so that should one species give out they can continue foraging. Consequently, the bumblebees' short learning flights when departing a flower may be part of a mechanism allowing them to be more flexible in the detection and exploitation of the resources in their environment. This strategy fits their more solitary foraging and the need, unlike honeybees, to decide for themselves where to forage.

Turning to more mechanistic questions, the results of tests in which the cylinders were displaced revealed that despite longer learning flights, the bees were no more accurate in their

search for their nest than in their search for the flower. Longer learning flights in these experiments did not seem to improve the bees' ability to pinpoint a location. Would a difference have been seen if the flower were more inconspicuous? Might bees then perform longer learning flights at the flower or fail to learn its position? This needs further examination. Initial attempts were not successful because the bees did not return. More research is needed to say whether the attempts were inadequate or a genuine result. Although bees were equally accurate in their searches for the flower and the nest, they were more persistent in searching for the nest than the flower and their persistence at both locations tended to grow when the flower or the nest hole was marked by a purple ring. Variations in persistence could be a consequence of the value of the goal – the nest being of higher value than the flower with a longer inbuilt search linked to the nest than to a flower. Or the persistence could be a direct consequence of the strength of the memory. In this case the enhanced synaptic transmission in visuo-motor circuits would lead to slower habituation. Indeed, the process of the learning flights may strengthen the synaptic connections in the relevant neuronal circuits proportionally to the learning flight duration in the form of long term potentiation (Nicoll, 2016). At the scale of one attempt to return at the nest, because the long term potentiation increases the presynaptic release of calcium and causes more neurotransmitter receptors to be inserted in the postsynaptic membrane, the concerned synapses will respond for a longer duration before habituation (Gluck et al., 2013). On a larger scale, because of the extended duration of the learning flights at the nest, the depotentiation of these synapses may take days, and thus the bumblebees will persist in regularly coming back to search for their nest for an extended period of time.

Chapter 3

How the value of a reward influences the learning flights of bumblebees on leaving a flower

Abstract

Bumblebees, *Bombus terrestris*, explore and forage within an uncertain floral environment. When they encounter a profitable flower, they perform a learning flight in which they turn back to fixate the flower, acquiring information that can guide their return to it. We investigated here how a change in reward value modulates these flights. First, we asked first whether the duration of drinking and the duration of learning flights change with sucrose concentration. We recorded bumblebees in a greenhouse and inside a flight room when they left a stationary artificial flower. The flower contained sucrose of either 10%, 20%, 30% or 50% w/w. Bees left their nest singly and were caught and placed gently on the flower to feed ad-libitum. Drinking duration was positively correlated with the volume of sucrose solution they drank. Bees tended to drink for longer when the concentration was higher (e.g. 50%) than when it was lower (e.g. 20%). The duration of learning flights increased significantly with sucrose concentration, suggesting that that the bees invested more effort in learning the location of the flower when rewards were higher. We then asked what happens to the duration of learning flights when the 20% concentration is raised after the third visits to the flower. We predicted an increase in learning flight duration, if bees would attempt to strengthen the association. During the first three visit, the duration of the learning flight became shorter. On the bees' 4th visit the concentration was increased to 50%. Although the bees spent longer drinking, indicating that they had noticed the change in concentration, their

learning flight did not become longer. These results suggest that once bees have learnt the location of a flower, enhancing its reward value does not re-elicit learning flights.

3.1 Introduction

In the previous chapter (Chapter 2), I have shown that the bumblebees perform shorter learning flights when departing a flower than when departing their nest. To explain this phenomenon, one of the proposed explanations is that the bumblebees adapt their investment in their learning flights to the value (or importance) that the location has for them. Because the nest is unique and that it is critical for the foragers to be able to bring back food to the colony, the bumblebees would invest more in learning its location than the location of a non-unique and transient flower. The question arises whether and to what extent the bumblebees would adapt their investment in their learning to the value of different flowers.

Indeed, in the wild, foraging honeybees and bumblebees encounter a wide array of flowers dispensing nectar of varying quality and quantity and are able to learn which flowers are most rewarding and to concentrate on visiting those. When bees forage among artificial flowers of differing colours, their preference is driven more by the quality than the quantity of the sucrose available. This preference hierarchy corresponds to the natural situation in which rewarding flower species will be competed for and those encountered will often be partially depleted. In this case, other flowers nearby of the same species are also likely to contain high quality nectar. Bees have a movement pattern that matches this scenario: bees on leaving a flower yielding a high reward tend to fly a short distance in the expectation of finding another flower of the same species in the same patch, and fly further away after leaving a flower that is depleted or is of low quality (e.g. Heinrich, 1979; Waddington 1980, Keasar et al., 1996). Honeybees also drink less from artificial flowers containing low than high concentrations (Núñez, 1966, 1970). Drinking less and flying further suggests the implementation of a

strategy of exploring for better flowers elsewhere. Shifting does not always occur. Thus, honeybees having learnt the location of a food source that drops in value may indicate the drop by ceasing to dance on their return to the hive, but still revisit the source, balancing the drop in value against the difficulty of locating a better source. Studies of this kind indicate that bees learn to associate the visual and olfactory cues of a flower with its value and that a complex and variable suite of behaviours can ensue. Here we focus on the learning flights that bumblebees (*Bombus terrestris audax*) perform when they depart after drinking from flowers of different concentrations.

Bees, like wasps, learn how to return to significant location, such as their nest or a foraging site, by performing learning flights on their first departures from the site (e.g. Tinbergen, 1932; Zeil and Collett, 1996; Zeil et al., 1996; Collett et al., 2016). In bumblebees, the flights consist of an elaborate set of manoeuvres during which bees learn both a route back to the nest and a panoramic view seen from the site that allows them to return over a broader range of directions (Hempel de Ibarra et al., 2009; Philippides et al., 2013; Collett et al., 2013; see also Chapter 2). Learning flights when leaving a flower are not only concerned with learning the location of the flower, but also with the flower's colour and shape. Indeed, although flower colour can be learnt on approach (Opfinger, 1931; Menzel, 1967; Lehrer, 1993), it is only on departure that the bee can know the value of what has been learnt on arrival and with this knowledge decide where to forage next.

Wei et al. (2002) asked whether the reward quality of a flower influences the length of learning flights. They trained honeybees to visit a feeder containing 0.5mol/l and after about 10 visits they shifted the reward to another location. The length of the flights in the new location were longer when the feeder contained 1.0mol/l at than when it contained 0.5mol/l. In a later paper, Wei and Dyer (2009) obtained evidence that longer learning flights enabled

bees to locate a feeder more easily suggesting that longer flights can result in the formation of 'better' memories.

We wished to know in more detail how learning flights might change with the reward offered by a flower. To do so, we recorded the learning flights of several groups of bees. Each group was presented with a different concentration of sucrose contained in an artificial flower. We first asked whether bumblebees (*Bombus terrestris L*) drink more and for longer with increasing sucrose concentration and how drinking time translates into the amount of sucrose drunk. We then compared the length of learning flights associated with differing concentrations of sucrose and analysed the flights for differences in several flight parameters. We also examined return flights to the flower to see whether approach flights also differ with sucrose concentration. Our final question concerns what bees learn once they have made several visits to the same flower and are accustomed to it. Whilst Wei et al. (2002) and Wei and Dyer (2009) presented rewards at variable locations, here the bee was confronted with the flower in just one location. Would the learning flight duration increase, and resemble the effect that an increase of sucrose concentration has on the waggle dances of honeybees (von Frisch and Jander 1957, Seeley and Visscher 1988, de Marco and Farina 2001)?

3.2 Materials and Methods

Experiments on five commercially reared colonies of bumblebees (*Bombus terrestris audax*, Koppert UK) were conducted between May and August 2016. Colonies were given pollen in the nest and, except during experiments, they were also provisioned with sugar syrup. Most experiments were run in a greenhouse (8 by 12m floor area) on the University of Exeter's Streatham Campus. Experiments on one colony, in which bees were weighed before and after they had fed at an artificial flower, were performed in a temperature-controlled laboratory

room (3.5x5m, 3.5m height, 21deg air temperature) in December 2016 with high contrast patterns on the walls.

3.2.1 Experimental procedure in green house

Each colony was placed beneath a table (1.5x1.8m, 1.5m height) with the nest-box connected to a hole in the centre of the table via a series of tubes. The arrangement allowed the experimenter to control the release and return of individual bumblebees. An artificial flower was placed on a second, similar-sized table about 5 m away. The flower was a flat, purple plastic ring (5 cm outer diameter) with a small transparent centrifuge tube containing sucrose in the middle (see also Chapter 2). Both tables were covered with white gravel that was frequently raked. Three black cylinders (17cm high x5cm wide) were placed equidistantly around the flower in a 120^{o} are at a radial distance of 24.5 cm from the flower (see also Chapter 2).

Workers were released singly from the nest. We selected by eye the larger individuals amongst the bees that left the colony and were in an exit box that was attached to the colony. Each bee performed a learning flight and was then caught and placed on the artificial flower, where it drank sucrose solution *ad libitum* (see also Chapter 2). The bee's learning flight on departure from the flower was recorded with a video camera (Panasonic HC-V720, HD 1080p, 50 fps) that was hung 1.35m above the table. The flower was cleaned and filled with fresh sucrose solution just before each bee was released.

To examine the relation between sucrose concentration and learning flights on leaving the flower, we recorded the first learning flight of new foragers after they were placed on the flower and had finished drinking. Each bee encountered one concentration of sucrose in the flower (10%, 20%, 30% or 50% w/w). The order of concentrations was varied across

successive days of the experiment. After this one learning flight, bees were caught and not used again.

To determine whether bees increased the length of their learning flights after sucrose concentration had been raised, bees performed five complete foraging trips between their nest and the flower. They were placed on the flower containing 20% sucrose w/w after their first learning flight from the nest. On subsequent visits the bees found the flower on their own, apart from two bees that had to be helped to find it on their second visit to it. The concentration was kept at 20% for the bees' first three visits to the flower. On the bees' fourth and fifth visits to the flower, the sucrose concentration in the flower was raised to 50%. The bees' drinking times and their learning flights on departure from the flower were recorded. For the analysis of return data from these bees rewarded with 20% with returns of bees that were rewarded with 50% at the flower, we referred to data from an experiment conducted between June and September 2015 in the same greenhouse (see Chapter 2).

3.2.2 Experimental procedure in the laboratory

The procedure when measuring the bees' weights were mostly the same as above, except for a few details. The room was too small to accommodate two tables. Consequently, the nest was kept on the floor in one corner of the room. The table with the artificial flower was the same size as in the greenhouse, but covered with a white cotton-loop bath mat (M&S, UK, see also Hempel de Ibarra et al. 2009) rather than gravel. The walls of the room were lined by a high-contrasting black-white and red-white patterns to provide optic flow and visual cues so that the bees could regulate their flight normally (as used by Linander et al. 2016). Each bee was weighed with a precision weighing balance (Ohaus PioneerTM, USA) and then placed on the flower where it found either 20% or 50% w/w sucrose. The sequence of reward presented at

the flower was pseudo-randomised. After its learning flight on leaving the nest had been recorded, the bee was reweighed, labelled for future identification and frozen. The size of each bee as given by the width of the thorax was measured later under a dissecting microscope using a digital caliper (Axminster, UK).

3.2.3 Data analysis

Videos were scored using video editing software (Adobe Professional Suite). To measure the duration of learning flights on leaving the flower, we noted the time interval between the bee starting to fly and when it first reached a radial distance of 24 cm from the flower. The duration of drinking was measured with the bee's image magnified so we could record when a bee inserted and retracted its proboscis into or out of the sucrose. Sometimes the bee tended to lean forward, making the proboscis impossible to see. In this case, we took as drinking duration the interval between the bee stopping moving after placement on the flower and the bee's slight movements that indicated proboscis withdrawal. The durations of any breaks in drinking were subtracted from the overall drinking time.

To analyse the details of learning and return flights, we extracted the bees' positions and body orientations during each flight using custom-written codes in Matlab (Philippides et al. 2013). From the bees that drank 20% or 50% we selected for analysis 10 bees from each group, with flight durations close to the group's median. Flight variables were analysed in R (version 3.2.0). Trajectory lengths are taken as the cumulative distance that a bee travels, before it crosses for the first time a circle of a given radius. Similarly, durations are taken as the time a bee travels before crossing a circle of a given radius.

A significant part of learning flights is when the bee turns back and fixates its point of departure. To analyse such fixations of the flower, we scanned each flight to extract groups of at least 4 successive frames in which the bee’s orientation relative to the flower varied no

more than 3º (see Chapter 2) for a detailed account of the extraction of fixations). For some figures we segregated these fixations into those in which the bee fixated the flower itself, that is the angle between the line connecting the bee to the flower and the bees' longitudinal axis was less than 20º and those fixations in which that angle was greater.

Statistical tests on the data were performed using IBM SPSS Statistics (version 23) and R (version 3.2.0).

3.3 Results

3.3.1 Sucrose concentration, amount drunk and body weight

Because experiments of this type are new for bumblebees, we determined whether bumblebees drink more from artificial flowers dispensing 50% w/w than from those containing 20% w/w sucrose solution. We also determined how drinking time, which in the following experiments we take as a simple proxy for drinking volume, is related to the volume of sucrose ingested. We estimated a bee's drinking volume from the difference in its weight before and after feeding and have plotted drinking time against drinking volume (Figure 3.1). The two parameters are strongly correlated and both measures indicate that bees consume more 50% than 20% sucrose (Figure 3.1). Similarly, the learning flights of the 50% group were significantly longer (Median duration 6.3s, IQR=3.9, n=18 bees) than that of the 20% group (Median duration 3.7s, IQR=2.09, n=23 bees; Mann-Whitney U=105, Z=-2.68, p=0.007).

In all experiments, we selected larger bees by eye. To ensure that our selection procedure was reasonably standardised and that differences between groups are unlikely to be attributable to

differences in the size of the bees in different groups, we also measured the bees' body size as given by thorax width. There was no size difference between the sample of bees drinking 20% sucrose (Median thorax width 4.99mm, IQR=0.33, n=23 bees) and the sample drinking 50% sucrose (Median thorax width 4.95, IQR=0.66, n=18 bees, Mann-Whitney U=188, Z=-0.499, p=0.62). A direct test showed that learning flight duration was not correlated with body size (Spearmans 20% r_S=-0.39, p=0.07, n=23 bees; 50% r_S=0.21, p=0.4, n=18 bees; both groups r_S=-0.031, p=0.85, n=41 bees).

3.3.2 Learning flights and sucrose concentration

Bees were placed on artificial flowers filled with sucrose at concentrations of 10%, 20%, 30% or 50% (w/w) and their learning flights recorded when they left the flower. The bees' median flight duration before bees reached a radial distance of 24 cm from the flower was related to the concentration of the sucrose that they had drunk (Figure 3.2). Example flights (Figure 3.3) suggest that there are not large differences in the overall form of the flights. Nonetheless, more detailed analysis reveals significant but subtle differences between the lower and higher concentrations. To gain a better appreciation of how bees distribute their time as a function of their radial distance from the nest, we analysed the path length and duration of the bees'

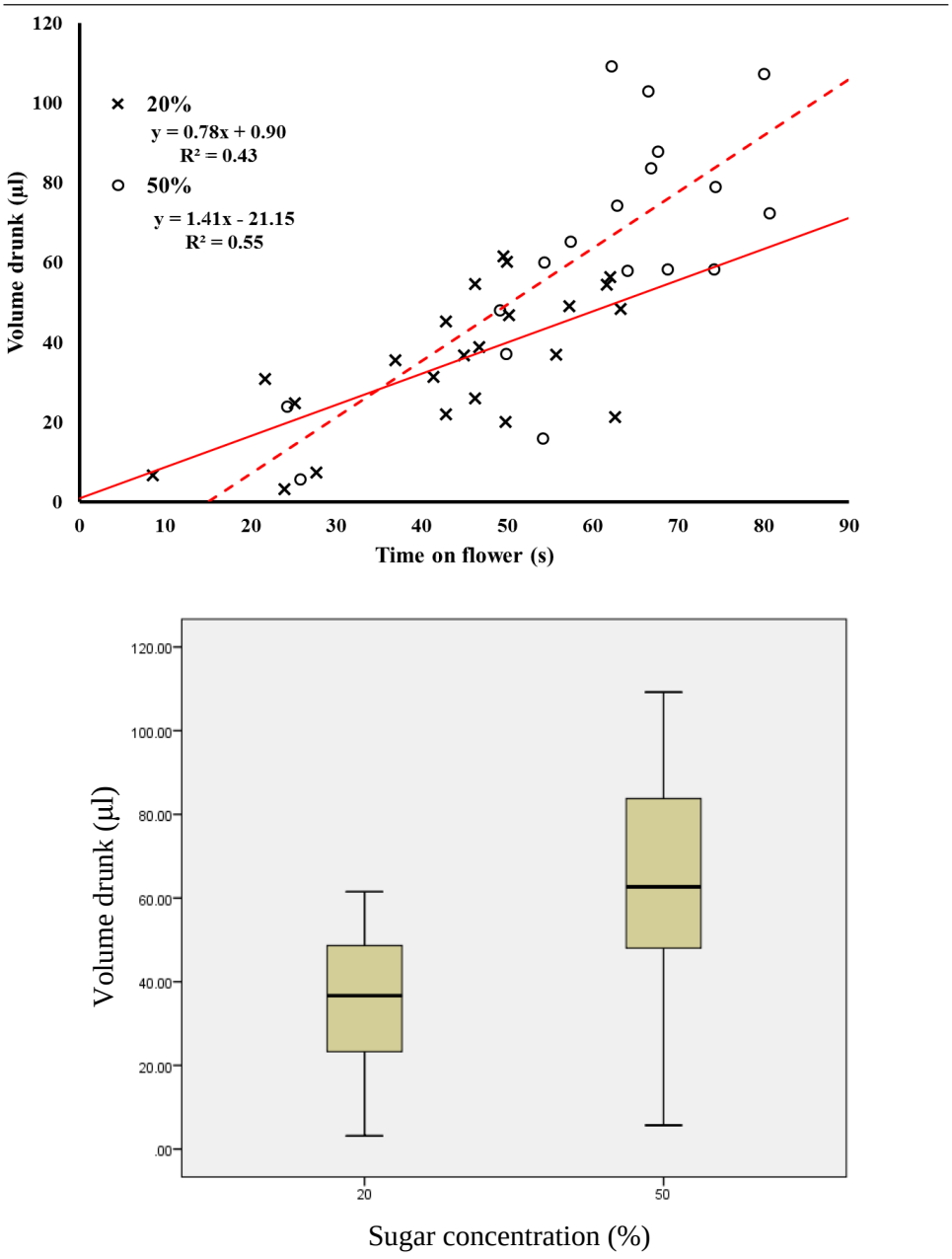


Figure 3.1. Volume drunk and drinking time when inexperienced bumblebees encounter a flower for the first time. (A) The bees' drinking time is correlated with the volume that they drink (20%: ρ=0.66, n=23, P=0.001; 50 %: ρ=0.74, n=18, P<0.0001) and both drinking volume (20%: median 37.46, IQR=27.74; 50%: median 55.90, IQR=35.22, t(39)=2.969, p=0.005) and drinking time (20%: median 46.36, IQR=18.84; 50%: median 63.47, IQR= 17.05, t(39)=3.340, p=0.002) are greater for bees given 50% sucrose than for those given 20% sucrose. (B) Bees drank larger volumes when rewarded with 50% than with 20%

(Median (20% volume drunk)=36.6µl, IQR=27.13; Median (50% volume drunk)=62.66µl, IQR=39.48; Mann-Whitney U=84, Z=-3.23, p=0.001).

learning flights before they first crossed a succession of circles centred on the flower, with the radius increasing in 2 cm steps (Figure 3.4A, B). The bees' flight duration does increase with the sucrose concentration of the flower (Figure 3.4A), but trajectory length is too variable to be sure (Figure 3.4B).

The bees' learning flights when leaving the nest seems to consist of two distinct phases. During roughly the first third of the flight bees, stay close to the nest, within about 5 to 6 cm from it. In the second two thirds of the flight, the bee gradually increased its distance from the nest, periodically returning all or part of the way to the nest. When we divided each flight into equal time intervals of a tenth and then took the median distance for each of the ten bins, it was clear that the same one third, two thirds relationship holds when bees leave flowers with different concentrations (Figure 3.4C).

During learning flights when leaving either the nest or the feeder (Chapter 2), bees tend to fixate for brief periods the location that they are leaving, suggesting that during these fixations bees may acquire views of these locations and their surroundings that can guide later returns. Fixations relative to the feeder were extracted (see Methods and Chapter 2). As sucrose concentration dropped the bees' tendency to fixate the flower became weaker (Figure 3.5). The peak of the distribution of fixations is centred on the flower in flights performed after feeding on 50% sucrose. After bees have drunk 20% or 30% sucrose, the variance of the distribution is greater and the peaks less prominent and not focussed on the flower. All fixations relative to the flower across all three concentrations occur mostly when the bees are close to the flower (Figure 3.6). In part this unequal weighting close to the flower is just a consequence of bees spending a disproportionate time close to the flower during the first phase of the flight.

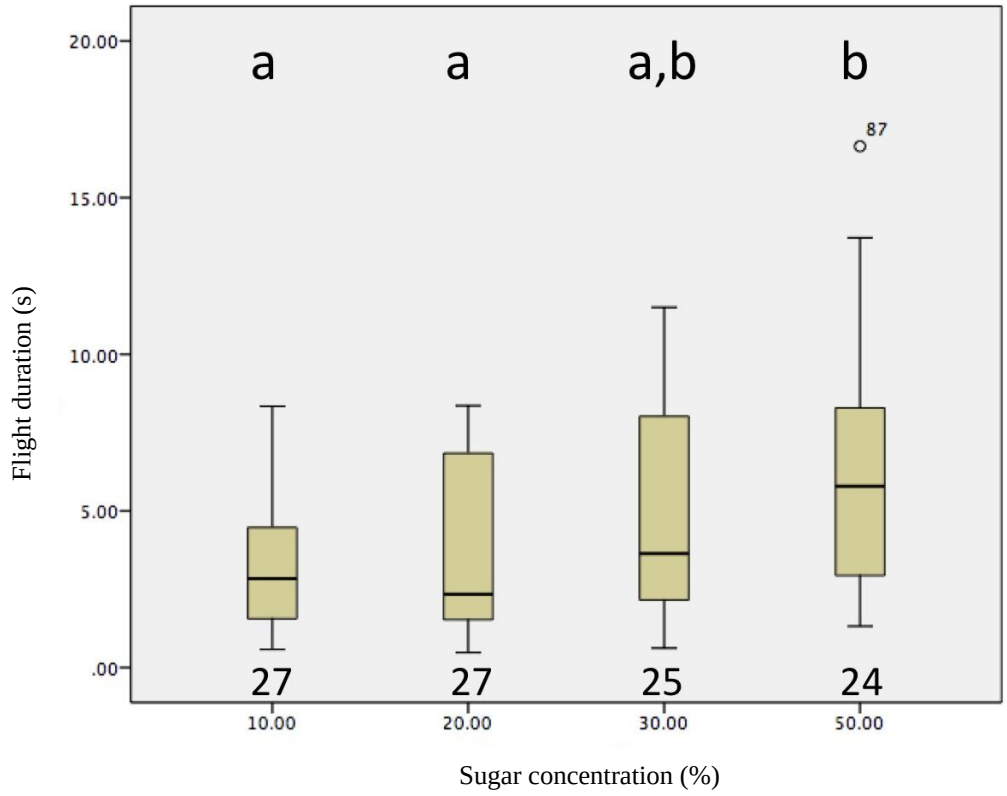


Figure 3.2. Durations of learning flights when departing a flower. A different group of bees fed at flowers of one of four concentrations (10%, 20%, 30%, or 50% w/w). A one way ANOVA shows a significant difference across all groups $F(3)= 4.893$; $p= 0.003$. The duration of the learning flights on leaving the flower differed significantly between the three lower (10%, 20%, 30%) and the two higher concentrations (30%, 50%), but not within the two lower or two higher concentrations (post-hoc Tukey HSD, $p<0.05$).

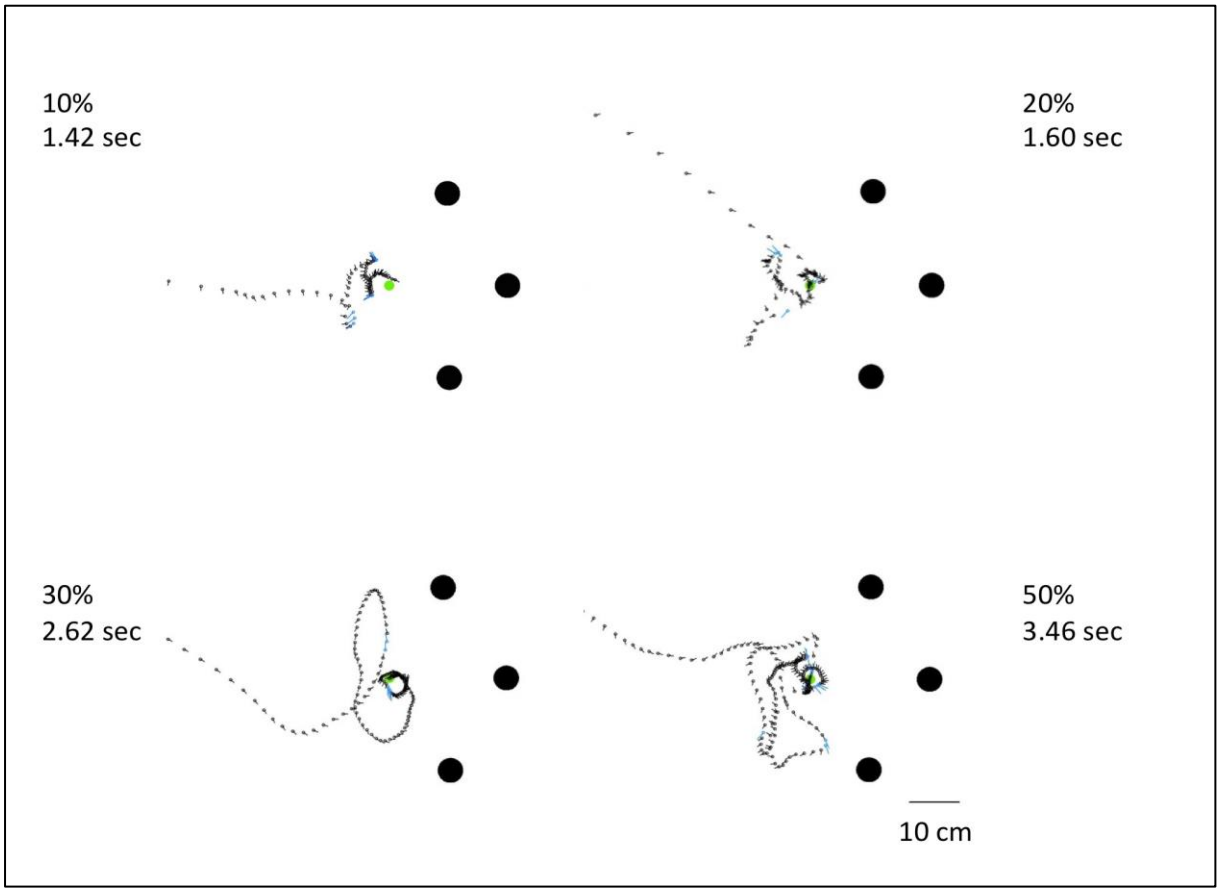


Figure 3.3 Selected examples of learning flights. Each point depicts the position of the bee at every second frame, i.e. every 0.04s. The line indicates the body orientation of the bee. The location of the flat pink artificial flower (5cm in diameter) is shown as a green dot. The three black dots represent the three black cylinder surrounding the flower. The concentration of the reward imbibed prior to the learning flight (percentage, w/w) and the duration of the learning flight are shown for each example.

Some features of fixations do not vary with concentration (Figure 3.7B). Thus, the overall rate of making fixations (number of fixations/number of frames in the flights) is similar for all three concentrations (20%: 0.047 ± 0.01 (Median); 30%: 0.04, IQR=0.02; 50%: 0.04, IQR=0.012). But for bees drinking the two higher concentrations, their rate of flower fixation plays a large role. If we focus on fixations of the flower when the bee's body is oriented within ±20^{o} of the centre of the flower, the rate of fixation is higher at the start of the flight (Figure 3.7A) when the bee is 5 to 7 cm from the flower centre (Figure 3.7C). In flights from bees drinking the weakest concentration of sucrose (20% wt/wt), there were almost no flower fixations.

The rate of fixation for the residue of fixations relative to the flower (i.e. fixations with the body oriented more than 20^{o} from the flower) was noisy with no definite peak (Figure 3.7B, D). The differences in flower fixation with sucrose concentration suggest that bees feeding on a high concentration give themselves more opportunity to learn about the flower and its surroundings than do bees encountering lower concentrations.

3.3.3 Return flights and sucrose concentration

The differences seen during learning flights when bees leave flowers containing different concentrations of sucrose is not reflected in the bees' return flights to the flower. The task of returning to a conspicuous flower seems to be sufficiently easy that even a non-dedicated learner exposed to a flower giving a low concentration of sucrose performs successfully. The data on return flights come from two different experiments. One experiment which is presented more fully in the next section examined how learned flights are affected by

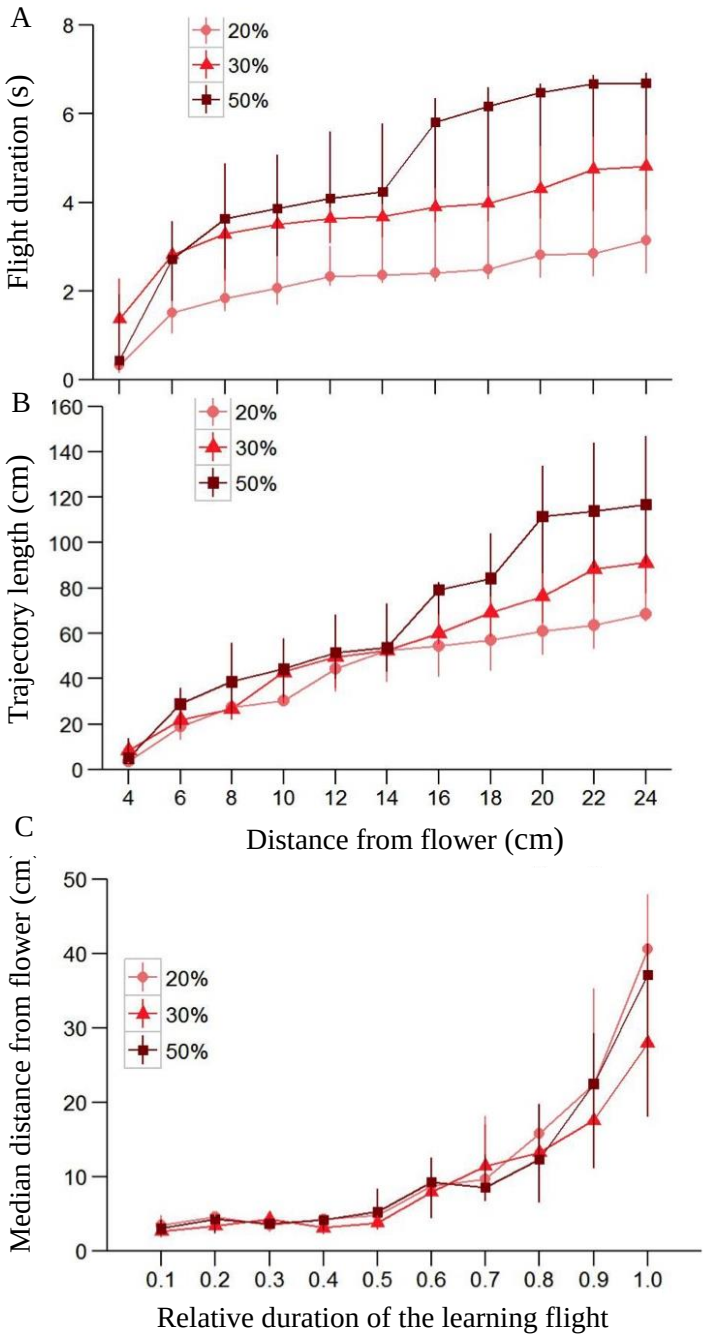


Figure 3.4. Effects on learning flights of different sucrose concentrations on the flights' duration, trajectory length and the bees' distance from the nest. (A, B) Cumulative median flight durations and trajectory lengths before bees first cross successive radial distances from the flower increasing in steps of 2cm to a maximum of 24 cm. (One way ANOVA $F(2)=7.107$, $p=0.003$) shows that duration of the flights increased with concentration. Post-hoc Tukey HSD shows significant differences between 20% and 30% ($P=0.017$) and between

20% and 50% (P=0.001). The trajectory lengths did not vary significantly with concentration (one way ANOVA F(2)=2.395, p=0.110). (C) Median distance from nest over the course of learning flights. Each flight to a maximum distance of 24 cm is divided into 10ths and a bee's median distance from the flower calculated over each 10th. Plot shows that these normalised distances are very similar for different concentrations (GEE χ^2=1.81, df=2, p=0.41).

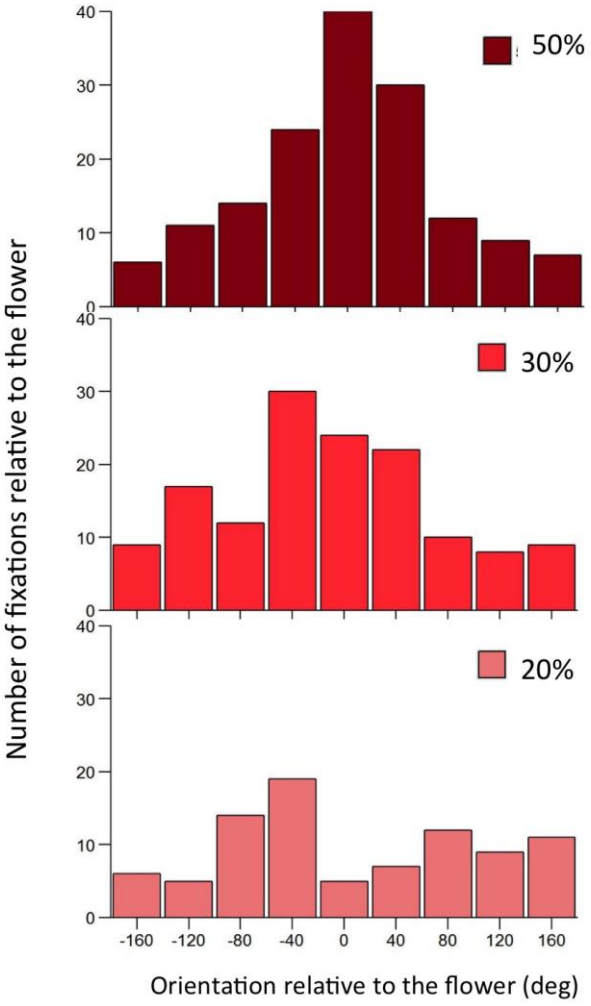


Figure 3.5. Properties of fixations relative to the flower associated with different sucrose concentration. Frequency distributions of fixations relative to flower. After drinking 50% sucrose the distribution of fixations during the subsequent learning flight is centred on zero (mode= 0º, circular variance=0.865). At lower concentrations, the mode is less prominent and the distributions have greater variances although, although the distribution of the 20% and 30% concentrations are not significantly different (30%: mode=-40º, circular variance=0.993; 20%: mode=-40º, circular variance=0.874; Watson-Wheeler test 20%vs30%: W=6, df=2, p=0.05; Watson-Wheeler test 30%vs50%: W=5, df=2, p=0.09; Watson-Wheeler test 20%vs50%: W=20, df=2, $p<0.001$).

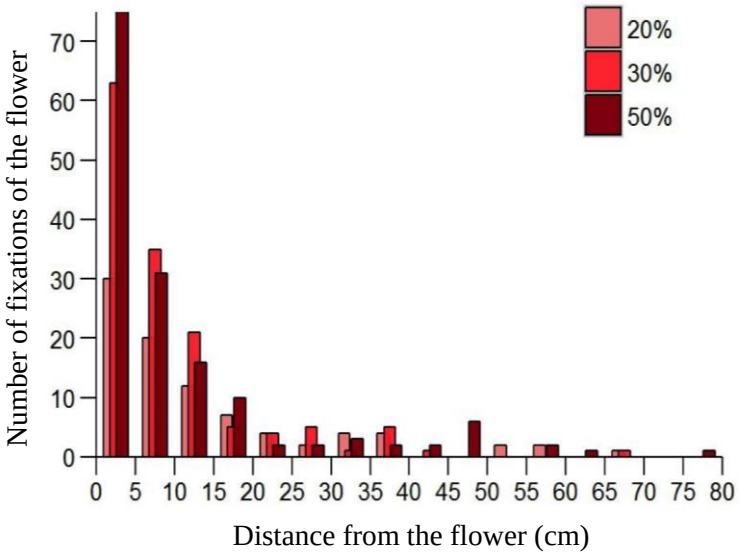


Figure 3.6. Number of fixations as a function of the bees' distance from the centre of the flower. For all concentrations, the majority of is within 10 cm of the flower. The preponderance is greater for the two higher concentrations but not significantly different (Kruskal-Wallis, $\chi^2(2)=6$, $p=0.06$).

increasing the concentration of sucrose from 20% to 50% after bees had made three return trips to the flower containing 20% sucrose. The second experiment, in which the sucrose concentration was 50%, compared the bumblebees' learning flights and return flights during successive foraging trips between their nest and a flower (see Methods). As learning occurs on approach flights as well as on departures, we only consider the first return flight to flowers of these two concentrations. Plots of the median distance and duration during the bees' approach to flowers containing 20% and 50% sucrose are similar (Figure 3.8 A, B). In addition, the bees' fixations relative to both flowers are strongly peaked in the direction of the flower (Figure 3.8C).

3.3.4 Learning flights after an increase in sucrose concentration

Do learning flights also lengthen if the concentration of sucrose is increased after several foraging trips? Bees made three trips to a flower with 20% sucrose and on the fourth trip, the concentration was increased to 50%. Bees drank for significantly longer on the fourth trip when the concentration was 50% than when it was 20% (Figure 3.9A). In contrast, the mean duration of the bees' learning flights dropped over the three departure flights from the flower containing 20% sucrose, consistent with earlier data on honeybees (Wei et al 2002) and bumblebees (Phillipides et al. 2013). The duration of learning flights did not increase when the concentration was raised to 50% (Figure 3.5B). Thus, although the bees appreciated the increased reward and drank for longer, the higher concentration probably did not induce them to learn more about the flower and its location.

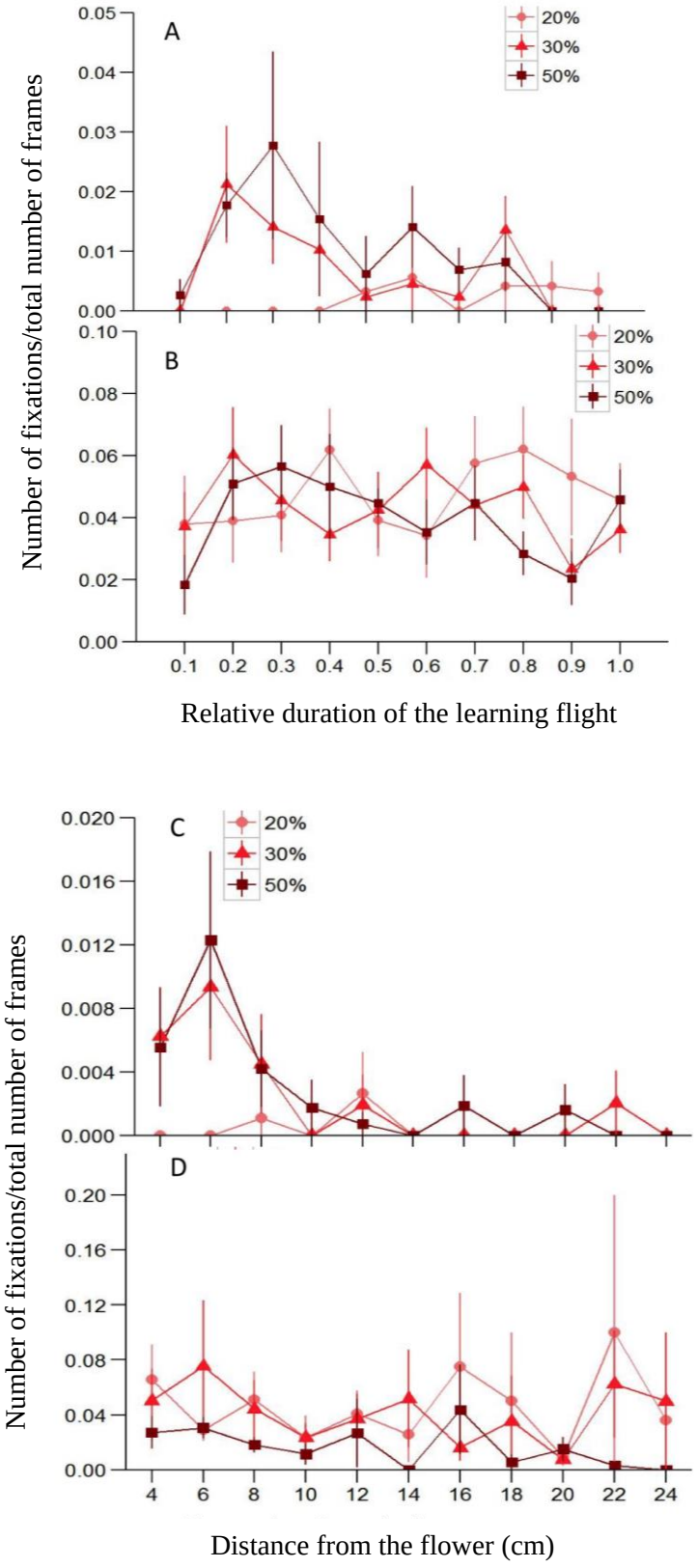


Figure 3.7. Rates of fixations relative to the flower associated with different sucrose concentrations. (A) Rate of fixations of the flower (±20^{o}) are plotted against relative time

during the flights. Fixation rates after drinking 50% and 30% sucrose peak within the first third of the flight. Fixation rates for 20% are low throughout the flight. (B) Rate of fixations in which the bees' body is oriented more than 20^{o} from the flower (C, D) As A and B but fixation rate is plotted relative to distance from centre of flower. Rates of fixations of flower after drinking 50% and 30% sucrose peak at 6 cm from flower. The residue of fixations (D) have no clear peaks for any concentration.

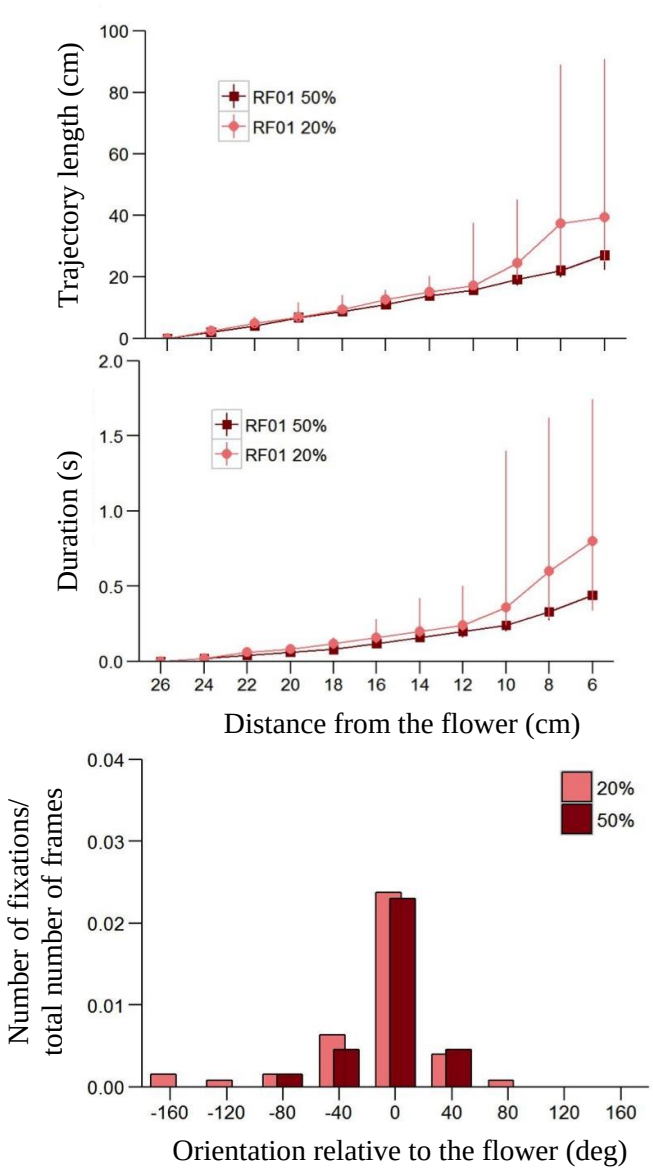


Figure 3.8. First return flights to flowers containing different sucrose concentrations**.** (A, B). Trajectory length and duration plotted as in Figure 3. There is no significant difference between returns to sucrose concentrations of 20% (N=10) and 50% (N=16). (Duration: Mann-Whitney, U=60, z=-1.78, p=0.08; Trajectory length: Mann-Whitney, U=70, z=-1.53, p=0.1). (C) Rates of fixation relative to the flower. The distributions for both sucrose concentrations peak when bee faces flower (20%: mode=0, circular variance=0.911; 50%: mode=0, circular variance=0.724; Watson-Wheeler test: W=1, df=2, p=0.5).

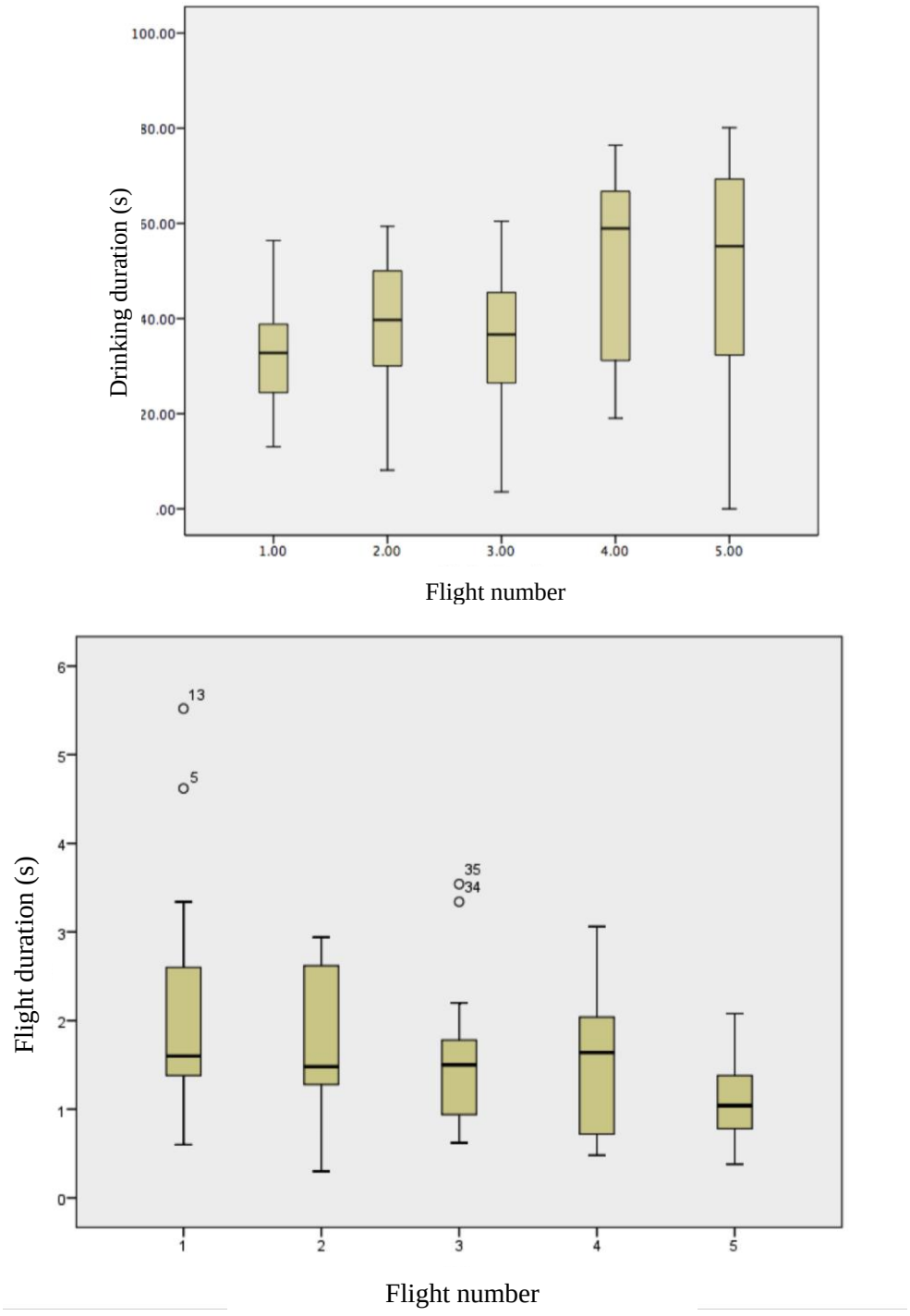


Figure 3.9. Drinking and learning flight durations when sucrose concentration is raised during a sequence of foraging trips for 13 bees that completed the whole experiment**.** (A) Drinking time during three visits to a flower when it contained 20% sucrose followed by two further flights after the concentration was raised to 50%. Drinking time rises significantly

between learning flights 1 to 3 and 4 to 5 (one way ANOVA 20%-50% F(1)=12.586, p=0.001). (B) Duration of learning flights after drinking. Duration of the flights drops significantly between flight 1 to 3 and flights 4 to 5 (one way ANOVA F(1)=5.022, p=0.029).

3.4 Discussion

In these experiments we have studied how learning flights alter with increasing reward level. Here we discuss how our findings relate to the function and generation of learning flights.
As has been reported earlier for honeybees, we found that bumblebees, *Bombus terrestris*, drink more from artificial flowers with relatively high sucrose concentrations (50% w/w) than from those with lower sugar concentration (20% w/v) (cf. Núñez, 1966) and that the duration of learning flights on leaving the flower increased when the reward was stronger (cf. Wei et al. 2002).
Do longer learning flights translate into greater success in locating a flower on a subsequent visit? Some evidence that longer learning flights do give bees a better opportunity to learn about a flower and its location comes from experiments by Wei et al. in 2002, in which returning bees were presented with a complex problem of feeder that changed position between visits. We gave bees the simpler task of just returning to a stationary flower and found that their first return after a single learning flight from the flower seemed as precise and as fast with a weak reward and shorter learning flight, as it was for higher rewards and longer learning flights. That suggests but may not prove that short learning flights are effective at least in the short term and for simple tasks. In future experiments, it would be worth taking two groups, one fed 20% sucrose and one 50% sucrose and give them a test them after their first departure flight from the feeder. In the test, the artificial flower would be removed and the array of cylinders shifted (see Chapter 2). Our expectation would be that the

50% group would search for longer and more precisely relative to the cylinders than the 20% group.

We went on to analyse the details of the shorter and longer flights associated with weaker and stronger rewards. What do the differences and similarities between the flights teach us about learning flights? The biggest difference that we spotted is in the frequency of flower fixations. Bumblebees increased their rate of facing and fixating the flower as reward quality grew. This increase was particularly notable at the start of a flight when the bees were only a few centimetres away from the flower (Figure 3.7).

Miriam Lehrer (1993) emphasised the importance of turning back and looking at flowers on departure from them. Looking back at a feeder proved to be an important feature of wasp learning flights as well (Collett and Lehrer, 1993; Collett, 1995). The present data reinforce the suggestion that flower fixations are significant for acquiring information about the features of a flower and its location. Fixations are not only more frequent with higher sucrose concentrations. They also occur at a higher rate. If we suppose that learning mechanisms are activated during fixations through increased activity of neuromodulators, then synaptic connections within the relevant circuits would be strengthened in proportion to the number of fixations. Stronger connections may lead to possibly longer lasting and more secure spatial memories (Nicoll, 2016).

Whereas the properties of fixations are likely to be relevant to memory processes, the characteristics of the learning flights, in which fixations are embedded, raise questions about motor control. Earlier observations (Philippides et al., 2013) were that, as a learning flight progressed and the bee moved further from, in this case, the nest, loops increased in size and in some examples maintained the same detailed shape. Collett et al. (2013) also noticed some fixed component of the bumblebees' learning flight. He highlighted the presence of straight segments in very specific compass directions, only few frames before the beginning of each

loop. He proposed that these straight segment represent the moment when the bees place themselves in a correct compass direction before to trigger a stereotyped flight manoeuvre that would place the insect in the correct position both relative to the nest and to the compass directions to memorise particular snapshots. However, until now, it was not known that some fix characteristics applied at the scale of the entire flight. Here, striking similarities between the learning flights after small and larger rewards are: 1) the constant overall rate of fixations relative to the flower (Figure 3.7) across 20, 30 and 50% sucrose and 2) the relative duration of the initial phase when the bee stays close to the flower which remains a fixed 0.3 to 0.4 of the flight across these concentrations (Figure 3.4). These constant characteristics point to a fixed spatio-temporal program of a learning flight that can be modulated in various ways and at different levels. These characteristics are reminiscent of motor skills, like human handwriting, in which an individual's style is constant across various transformations and which can be modelled in a hierarchical manner (Van Galen, 1991).

Remarkably, also in bumblebee flights we find an adaptive mix of fixed and flexible components, which here mediates the active process of acquiring visual information.

Chapter 4

Male bumblebees perform learning flights on leaving a flower but not when leaving their nest

Abstract

Female bees and wasps demonstrate, through their performance of elaborate learning flights, when and where they memorise features of a significant site. An important feature of these flights is that the insects look back to fixate the site that they are leaving. Females, which forage for nectar and pollen and return with it to the nest, execute learning flights on their initial departure from both their nest and newly discovered flowers. To our knowledge, these flights have so far only been studied in females. Here, we describe and analyse putative learning flights observed in male bumblebees *Bombus terrestris L*. Once male bumblebees are mature, they leave their nest for good and fend for themselves. We show that, unlike female foragers, males always fly directly away from their nest, without looking back, in keeping with their indifference to their natal nest. In contrast, after males have drunk from artificial flowers, their flights on first leaving the flowers resemble the learning flights of females, particularly in their fixation of the flowers. These differences in the occurrence of female and male learning flights seem to match the diverse needs of the two sexes to learn about disparate, ecologically relevant places in their surroundings.

4.1 Introduction

In many animal species, the two sexes, to some degree, know and learn about different things. In eusocial insects, like bees, the roles of females and males are quite distinct. In the bumblebee *Bombus terrestris L*., the species with which we are concerned here, a single female queen lays fertilised eggs. The other females are workers that perform one of several tasks for the benefit of the colony. They may, for instance, tend larvae, guard the colony, explore for nest or food sites, or forage for nectar and pollen, which they bring back to the nest. The workers forage individually and learn the locations of both their nest and good foraging sites. Male *B. terrestris*, in contrast, are not concerned about the state of the colony. They leave their nest to find potential mates and do not return (Goulson, 2010; Paxton, 2005). Indeed, possibly as part of a strategy to avoid inbreeding (Baer, 2003; Gerloff and Schmid-Hempel, 2005; Whitehorn et al., 2009), they may travel as far as 10 km from the colony, where they live as solitary foragers and patrol the terrain for queens (Coppée et al., 2011; Kraus et al., 2009; Paxton, 2005). Whereas males may well learn the location of foraging sites near to their patrolling area, they have no reason to learn to return to their nest. Are these differences in life style – in which females forage for the community and males forage only for themselves – reflected in the occurrence of learning flights in the two sexes?

Female wasps and bees on first leaving their nest or a flower perform elaborate flights (Brünnert et al., 1994; Collett, 1995; Collett et al., 2013; Collett and Lehrer, 1993; Hempel de Ibarra et al., 2009; Lehrer, 1993; Opfinger, 1931; Philippides et al., 2013; Stürzl et al., 2016; Tinbergen, 1932; Vollbehr, 1975; Zeil, 1993a,b) lasting sometimes tens of seconds during which they learn the location of that site. These learning flights consist of stereotyped manoeuvres (Collett et al., 2013; Philippides et al., 2013; Stürzl et al., 2016), which seem to be designed to pick up visual information that can guide their return to the site. The duration of flights when leaving a flower is probably related to the reward that the flower gives (Wei

et al., 2002; Wei and Dyer, 2009). But flights from the nest seem to be triggered by some appreciation of the nest's significance combined with the insect's lack of knowledge of the nest's location. So far, learning flights have not been studied in male insects. Some observations suggest that honeybee drones initially tend to perform short flights at the hive prior to mating flights, which could well be learning flights (Howell and Usinger, 1933; Witherell, 1971). Here, we first confirm earlier observations (Hempel de Ibarra et al., 2009), that male bumblebees depart directly from the nest without looking back, even though they have fed there. We then investigate whether the flights of males leaving flowers resemble those of female workers.

4.2 Materials and Methods

4.2.1 Experimental procedures

Experiments were conducted from June 2015 to March 2016 in a greenhouse (8x12 m floor area) at the Streatham campus of the University of Exeter. Male and female worker bumblebees, *Bombus terrestris audax*, from commercially reared colonies (Koppert UK), were marked individually with coloured number tags. *Bombus terrestris* is a ground-nesting species that leaves its nest through a hole in the ground. To mimic this situation, we placed each experimental colony under a table and recorded the flights of naive, male and worker bees when they left their nest through a hole in the centre of the table. We also recorded the bees' departures after they had fed from a flat artificial flower on top of another table (see below; Figure 4.1).

The tables (1.5x1.8 m) were covered with white gravel that was frequently raked. Three black cylinders (17x5 cm) were placed around the nest entrance at a distance of 24.5 cm (centre of the landmark). The nest entrance was surrounded by a purple plastic ring (5 cm outer diameter), which was frequently cleaned. A second identically arranged table with a sucrose dispenser (50% w/w) in the centre of another purple ring was placed 5 m away; we refer to the sucrose dispenser surrounded by the purple ring as an artificial flower.

Figure4.1. Experimental setup in a greenhouse: nest and flower tables. The nest table is in the foreground with the nest fixed under the table and its exit through a hole near the array of three cylinders. The flower table is in the background with the artificial flower in the same position relative to the cylinders as at the nest hole.

The behaviour of bees leaving the nest and the flower was recorded at 50 frames s^{-1} with video cameras (Panasonic HC-V720, HD 1080p) that were hung 1.35 m above each table. An area of approximately 70x90 cm was captured in an image of 1920x1080 pixels.

Male bumblebees (identified by the presence of claspers) typically emerge as adults once the colony is mature and after some days leave the nest. When a male bumblebee flew out of the nest, we let it fly around the greenhouse before catching it in a butterfly net. Because males were not motivated to feed for many hours after leaving the nest, they were kept overnight in a box and then placed individually on the flower the next day. We videoed males while they were on the flower and their subsequent departure from it. The data for workers came from a separate experiment. Worker bees were initially accustomed to artificial flowers by placing them individually on a similar flower on a third, training table. On their second flight from the nest, the training table was hidden and workers found the experimental flower on the second table by themselves.

4.2.2 Bees and colonies

We analysed data from 30 males from six colonies. After leaving the hive, the males were kept overnight in cages indoors and flew normally on the next day when leaving the flower or returning to it. The flights of 14 female foragers were recorded on their flights to and from the nest and flower. We analysed a bee's first departure from the nest and from the flower. Sometimes, departures from the flower were slightly aberrant, with the bee landing during these flights (n=5 workers, n=5 males); in such cases, we analysed the second departure instead of the first.

4.2.3 Data analysis

The positions and body orientations of the bees (Figure 4.2-4.4) were extracted from the video-recordings using custom-written codes in Matlab (Philippides et al., 2013). We define the duration of flights from the nest or the flower as the time it took the bees to cross a 30 cm radius circle centred on the nest entrance or the flower. Similarly, we define cumulative distance as the distance a bee travelled before it crossed a circle of a given radius for the first time. Both workers and males on their departure from the flower turned back to face and fixate the flower (Lehrer, 1993). Such fixations are typical of learning flights and we compared the fixations of the two sexes. To extract fixations relative to the flower, we first computed for every frame of each flight the angle between the bee's line of sight to the flower and its longitudinal body axis (ϕ; counterclockwise angles are positive; see Figure 3B). We then scanned successive frames of each flight, noting the modular angular difference ($\Delta\phi$) between adjacent frames, n and n+1. If $\Delta\phi$ was >3 deg, we repeated this calculation on the next pair of frames, i.e. frames n+1 and n+2, continuing the process until $\Delta\phi$ was $\leq$3 deg. Such a small rotational difference indicated the potential start of what we accept as a fixation. To test whether this was the case, we added the next frame to the two-frame sample. If the modular difference between the minimum and maximum values of the sample of three frames ($\Delta\phi$min.max) was $\leq$3 deg, we added the next frame to the sample and again tested whether $\Delta\phi$min.max of the four-frame sample was $\leq$3 deg. This loop was repeated, sequentially adding frames until $\Delta\phi$min.max of the whole sample was >3 deg. Provided that the sample size of successive frames with $\Delta\phi$min.max$\leq$3 deg was $\geq$4 frames, the sample was included as a fixation and we recorded its duration and the median value of ϕ. We then continued to scan neighbouring frames until we encountered the start of another potential fixation ($\Delta\phi\leq$3 deg), when once more we tested whether these and subsequent frames met our criteria for a

fixation. If they did not, the scanning of neighbouring frames was resumed from the second frame after the potential start. This process continued until the end of the flight and was applied to all male and worker learning flights at the flower.

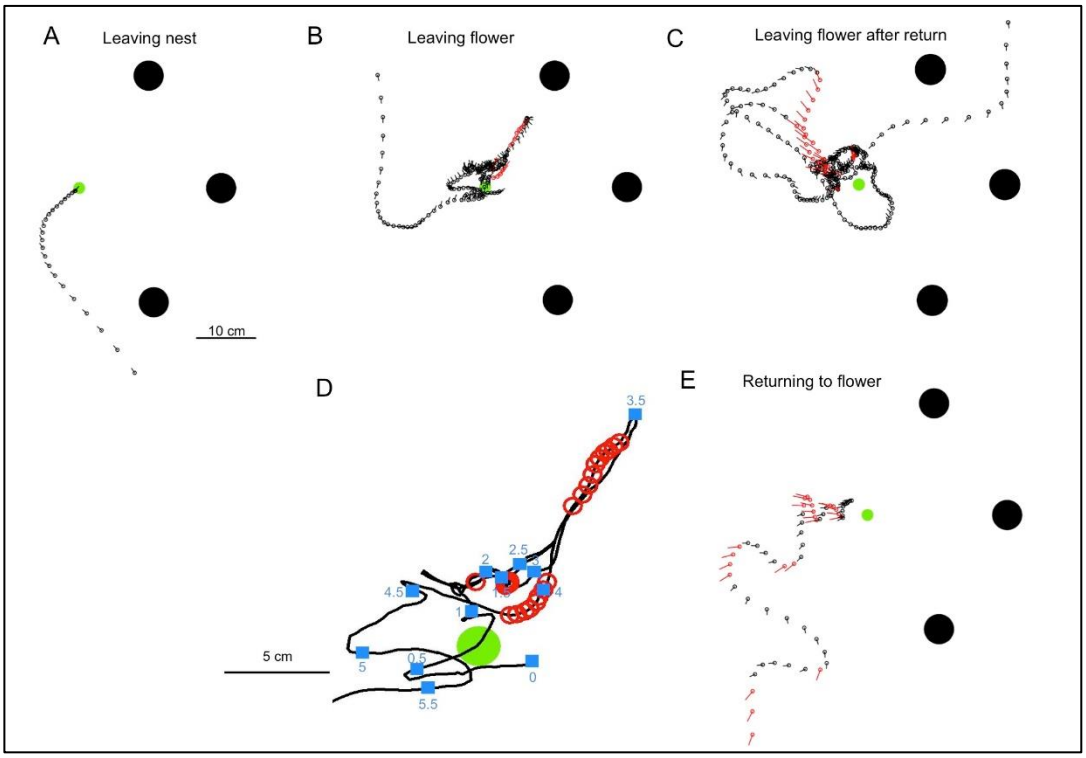


Figure 4.2. Example trajectories of male bumblebees. (A) Departure from the nest. (B) Departure from the flower. Flights in A and B are by the same male. (C) Departure from the flower by another bee after its spontaneous return to the flower. (D) Enlarged view of the initial segment of the flight in B. Red circles indicate fixations of the flower (see Materials and methods). Blue squares mark every 0.5 s from the start of the flight. (E) Return to the flower. In all panels except D, each dot shows the position of the bee every 0.04 s and each line shows the orientation of the bee's body. Red circles and lines indicate instances in which the bee faced the flower. Positions of the nest and flower are shown by a green circle. Black circles represent the array of three cylinders.

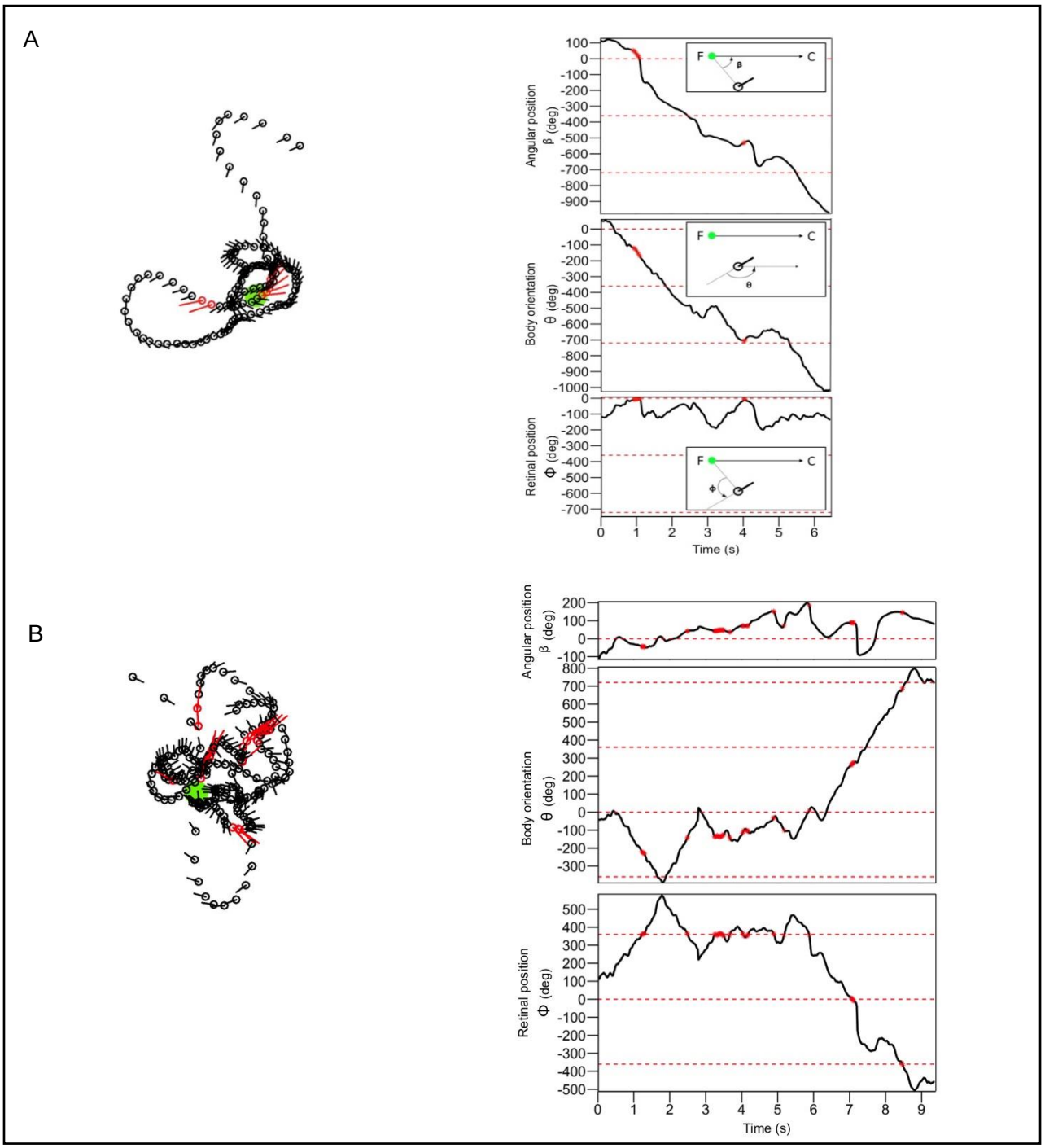


Figure 4.3. Further example trajectories of a male (A) and worker (B) leaving the flower. In some learning flights bees turn for long periods in the same direction, rotating clock- or counter-clockwise through several complete revolutions. In other flights bees alternate their direction of rotation. Each dot depicts the position of the bee every 40 ms. The line shows the bee's body orientation. Red circles and lines indicate when the bee faces the flower. Green circle shows the position of the flower. Time plots show the bee's cumulative angular position (β), its body orientation (θ) relative to the line between the flower and central landmark (F-C), and the position of the flower relative to the bee's longitudinal axis (ϕ, 'Retinal' position for short). Arrows (inset) point in a positive direction. Moments in which the bee faced the flower (±10°) are shown in red.

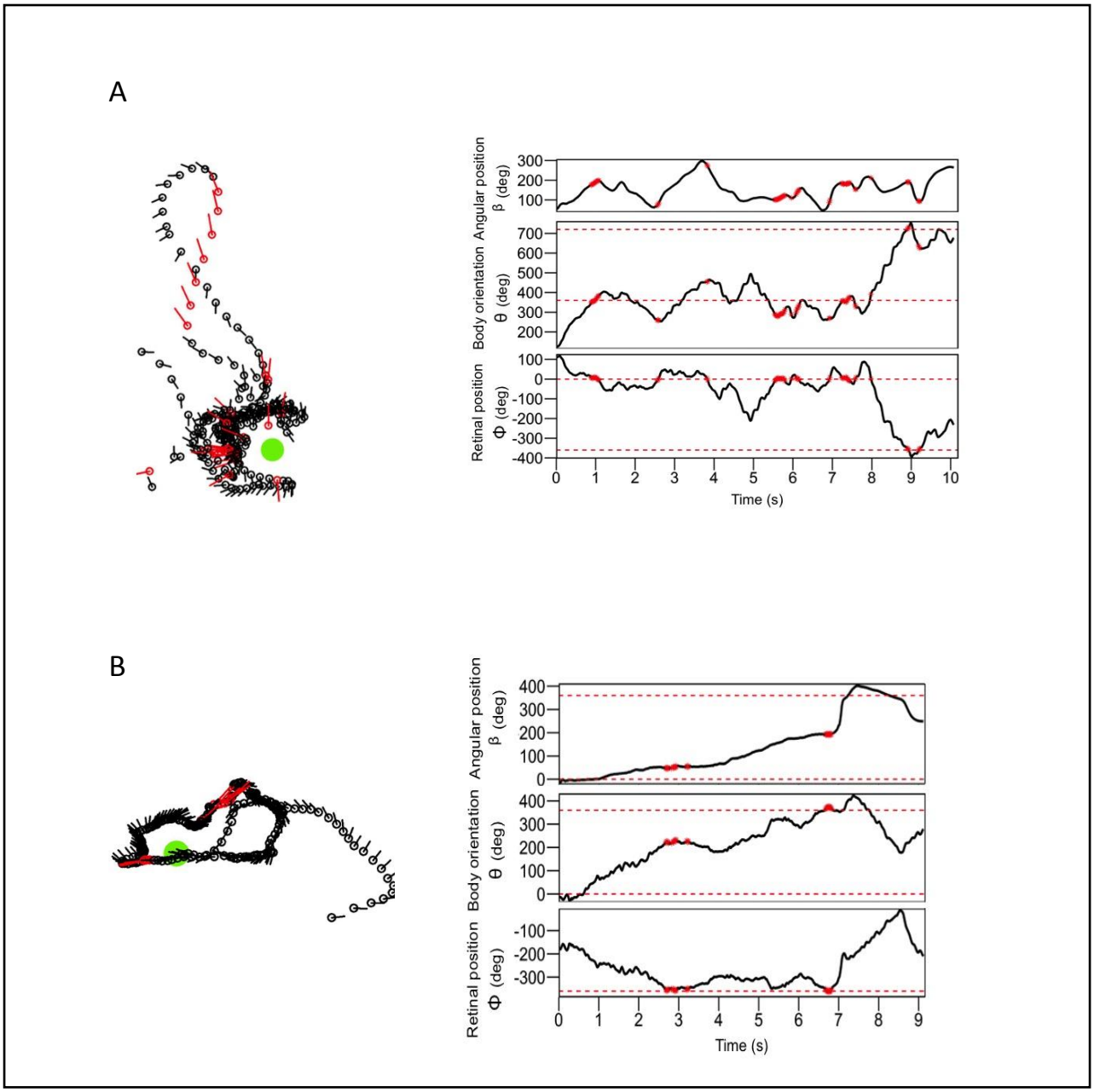


Figure 4.4. Further similarities in the learning flights of a bumblebee male (A) and worker (B) leaving the flower. For details see Figure 4.3.

To test whether the selected fixations were more precisely oriented towards the flower than would be expected from the overall distribution of ϕ across learning flights, we carried out two randomisation tests on male flights that were computed in R (version 3.2.0). In the first test, we combined all the frames of the measured fixation intervals into one sample. For each frame of this sample, we took the absolute value of the difference between ϕ and ϕ=0 deg and averaged these values. This total ('mean absolute difference'; see Figure 4.5) was then compared with the mean absolute difference of samples of the same number of frames, which were selected randomly from all the flights. We generated 100,000 random samples and found that none of the values for mean absolute difference of these 100,000 samples were smaller than the mean absolute difference of the real fixation sample (Figure 4.5). We therefore rejected the hypothesis that the flower-facing fixations can be obtained by randomly selecting frames from all the flights.

In the second test, we took each fixation separately and calculated the absolute value of the difference between the median ϕ of the fixation and ϕ=0 deg. These absolute values were averaged across all the fixations ('mean of the median absolute difference') and compared with that of random selections of consecutive frames. To do so, we randomly selected groups of consecutive frames from different flights to match the number of fixations and their durations (Figure 4.5) and calculated the mean of the median absolute difference of this random sample of groups. As in the first test, we repeated the randomisation procedure 100,000 times. Again, none of the values for the mean of the median absolute difference of these 100,000 samples were smaller than the mean of the median absolute difference of the real fixation sample (Figure 4.5).

4.3 Results

4.3.1 Departures from nest and flower

On their departure from the nest, males accelerated directly away without turning back to face the nest (Figure 4.2A). Their flights when leaving the flower were significantly longer and more complex (Figure 4.2B, D). The first departures from the flower were recorded when males were placed on the flower, rather than when they found it by themselves. To check whether this procedure might have disturbed the bees' flights on their departure, we tested workers with the same procedure. The workers' learning flights were of similar duration (see Materials and methods, 'Data analysis') whether they were placed on the flower (N=14, mean±s.e.m. 4.83±1.25 s) or flew there by themselves (N=14, mean±s.e.m. 5.33±1.02 s, Mann–Whitney U-test, U=78.5, Z=−0.90, P=0.37). This similarity suggests that the departure flights of males are also unlikely to be influenced by the way in which a bee reaches the flower.

Learning flights after a male's return to the flower support this suggestion (Figure 4.2C). Although males were deprived of food for about a day before being placed on the flower, the time that they spent on the flower was variable. Sometimes males flew away very soon and sometimes they stayed there for 2–3 min (Figure 4.6A). We had no direct measurement of when a bee started to drink and the time that it drank when on the flower, but it is reasonable to suppose that drinking time is correlated with time spent on the flower. As males forage for themselves and not for the colony, their motivational state may well be more variable than that of workers. The time that males spent on the flower was correlated with the duration of their flight on departure (Spearman Rank, rho=0.51, p=0.0042, Figure 4.6A), suggesting that a short time spent on the flower, allowing little or no time to drink, is insufficient to trigger

learning. For this reason, we excluded males that were in the bottom quartile of time spent on the flower (from 1.8 to 14.4 s) from further analysis. In several respects, the flights of males leaving flowers resembled those of females. The durations of the flights did not differ significantly (Fig 4.6B and C) in contrast to the very short flight durations of males leaving the nest and the very long flights of female workers when they left the nest. The similarities between the durations of male and female flights leaving the flower are mirrored in the cumulative distance plots (Figure 4.6D) in which both sexes took a longer, more circuitous route when leaving the feeder (Figure 4.2, 4.3, 4.4), than did males leaving the nest.

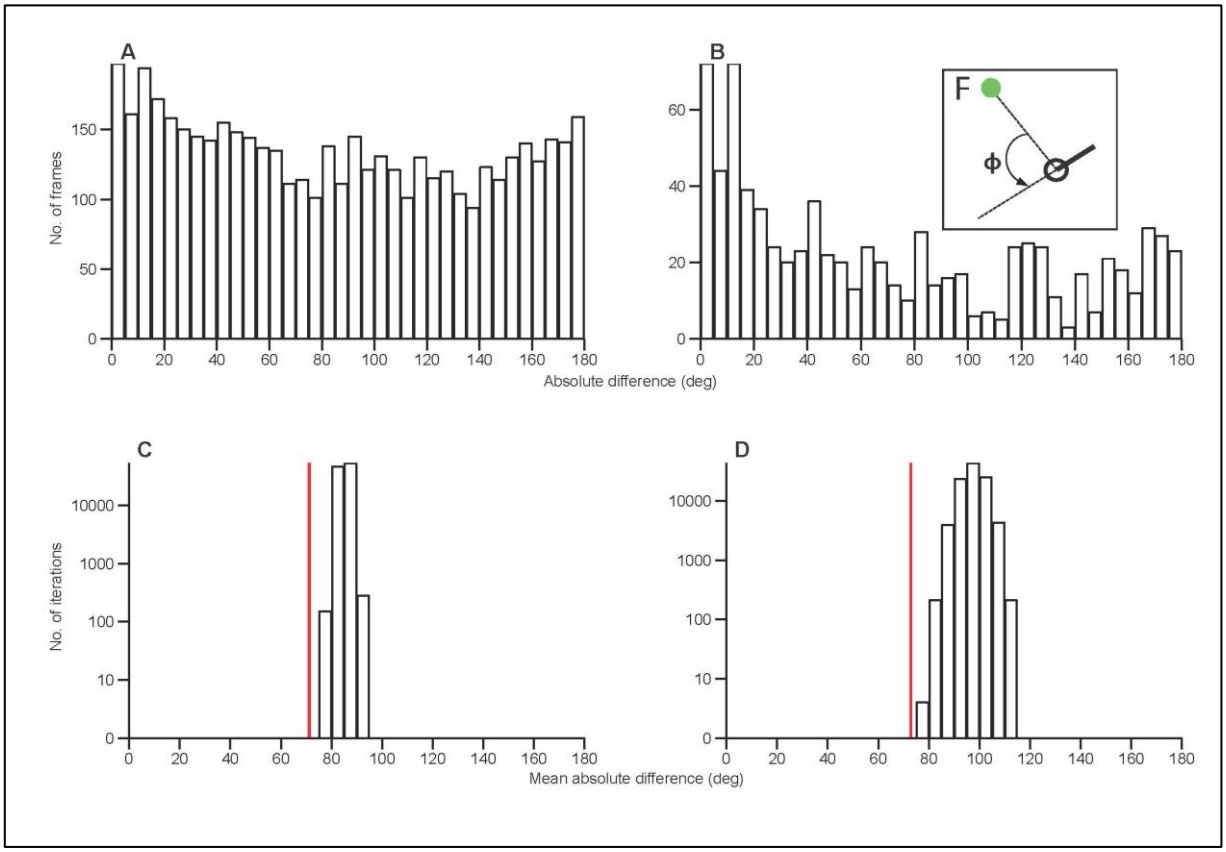


Figure 4.5. Analysis of flower fixations in male learning flights. (A,B) Distribution of the absolute values of angles between the male's body orientation and the line from bee to flower (|ϕ|) for every frame in every flight (4872 frames, N=24 males, n=24 flights; A) and for every frame in every fixation (821 frames; B). (C,D) Distribution of the mean |ϕ| of each randomly picked sample of individual frames (C) and groups of frames (D). In both cases, 100,000 samples were randomly selected. The red lines show the measured means of body angle orientation during fixations. Their position outside the distributions of the means of the randomly selected frames and groups of frames demonstrates that the measured distribution of body orientations in fixations is not a random selection from the overall distribution of body orientations during learning flights of male bumblebees. Inset in B shows the angle ϕ between the flower (F, green circle) and the bee's longitudinal body axis.

4.3.2 Flower fixations

A hallmark of learning flights is that bees or wasps look back towards the nest or flower (Collett and Lehrer, 1993; Hempel de Ibarra et al., 2009; Lehrer, 1993; Riabinina et al., 2014; Stürzl et al., 2016), presumably recording views that can guide their return. Whereas bumblebee males almost never faced the nest on leaving it, they, like workers, always looked back when they left the flower. The flight excerpt in Figure 4.2D includes three instances (marked in red) of a male facing the flower on departure. The first is a very brief period of hovering at 1.7 s. The second and third are longer, starting at 3.5 s and at 4 s. During the third, the male flies back towards the flower (see Figure 4.3 and 4.4 for further examples). This divergence in facing the flower but not the nest can be seen in plots of the body orientation of males relative to the nest and flower (ϕ) that include all the frames of all the recorded flights (Figure 4.7A). On flights from the flower, the broad peak of the distribution of facing directions relative to the flower (ϕ) is towards the flower circular mean: 44.02 deg, ρ=0.092, Rayleigh Z-test, Z=41.93, P<0.0001), but on leaving the nest, the peak of the distribution of facing directions is in the direction of departure (circular mean: 177.36 deg, ρ=0.726, Rayleigh Z-test, Z=615.38, P<0.0001): the bee faced the nest for only 0.35% of frames (Figure 4.7A).

To what extent are frames in which bees look at the flower grouped together so that the bees fixate the flower for consecutive frames? To examine flower fixations, we extracted periods when the direction in which the bees looked relative to the flower (ϕ) remained relatively constant (see Materials and methods, '*Data analysis'*). Partitioning all the frames of male departures from the flower into those that do and do not fall within these extracted fixations shows a strong peak towards the flower in the distribution of frames within the fixations (Figure 4.7B). No such peak is seen for the distribution of frames outside fixations. In addition, a resampling analysis (Good, 2006) shows that the distribution of frames in the

fixations has a significantly greater peak in the direction of the flower than would be expected from the overall distribution of frames from all flights (Figure 4.7A, see Figure 4.5 for details). It thus seems that bees tend to look at the flower during brief periods of fixation (Figure 4.7C).

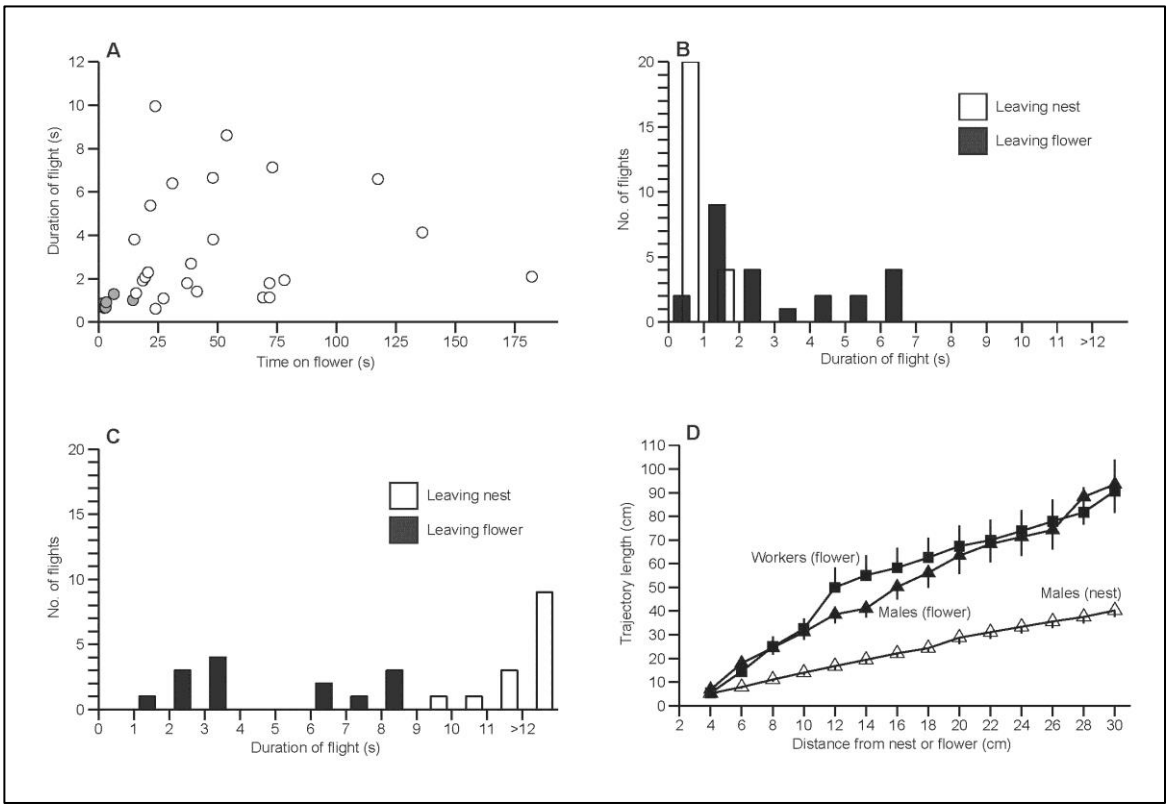


Figure 4.6. Some properties of male and female learning flights. (A) Duration of male departure flights from the flower (i.e. time taken to cross a 30 cm radius circle around the flower), plotted against time spent drinking on the flower before departure (N=30 males). Filled circles represent males with a short drinking time (bottom quartile) that were excluded from further analysis (N=6 males). (B) Duration of male departure flights from the nest and flower until crossing the 24 cm radius. (C) Duration of female departure flights from the nest and flower until crossing the 24 cm radius. The flights of males from the flower were a little shorter (N=24 males, n=24 flights, mean±s.e.m. 3.58±0.54 s) than those of workers (N=14 workers, n=14 flights, mean±s.e.m. 4.94±0.72 s) (Mann–Whitney U-test, U=109, Z=−1.79, P=0.07). Worker flights from the nest lasted longer than the other three categories, i.e. male flights from the nest and flower and worker flights from the flower (14.42±1.20 s). (D) Cumulative trajectory lengths flown by males leaving the nest or flower and by workers leaving the flower. Lengths at different distances from the flower or nest are those measured before the bee first crossed a circle of a given radius to a maximum of 30 cm. The mean±s.e.m. total trajectory length of males leaving the nest was 40.25±2.74 cm and that of males leaving the flower was 98.53±12.05 cm (Wilcoxon, W=21, Z=−3.69, P=0.0002). The mean±s.e.m. trajectory length of workers leaving the flower was 90.75±13.25 cm. It was

slightly but not significantly shorter than the trajectories of males leaving the flower (Mann–Whitney U-test, U=159, Z=−0.27, P=0.79). See also Figure 4.3 and4.4.

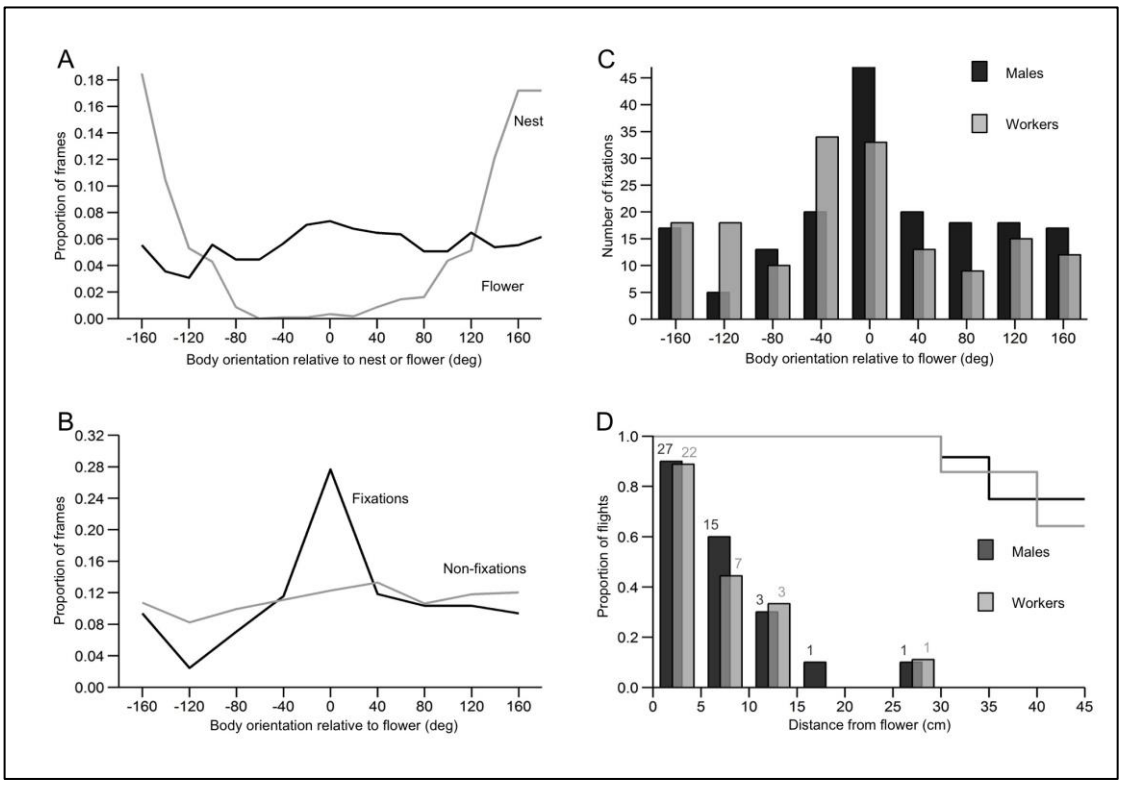


Figure 4.7. Fixations of flower and nest during learning flights. (A) Frequency distribution of male body orientation relative to the nest (grey line, 1169 frames) or flower (black line, 4872 frames; N=24 males) on departures from them. Bin width is 20 deg. (B) Distribution of body orientation relative to the flower when all frames of male departure flights are partitioned into those within fixations (821 frames) and those outside fixations (4051 frames). (C) Frequency distribution of male and worker fixations (175 male fixations, 162 worker fixations; N=24 males, N=14 workers) relative to the flower (40 deg bin width). (D) Proportion of flights with at least one flower fixation (ϕ=0±20 deg) within a specified distance from the flower (N=10 males, N=9 workers). The numbers above each 5 cm bin give the total number of fixations falling in that bin. The lines above the histograms show the proportion of flights at each distance that are at least as long as that distance (N=24 males, N=14 workers).

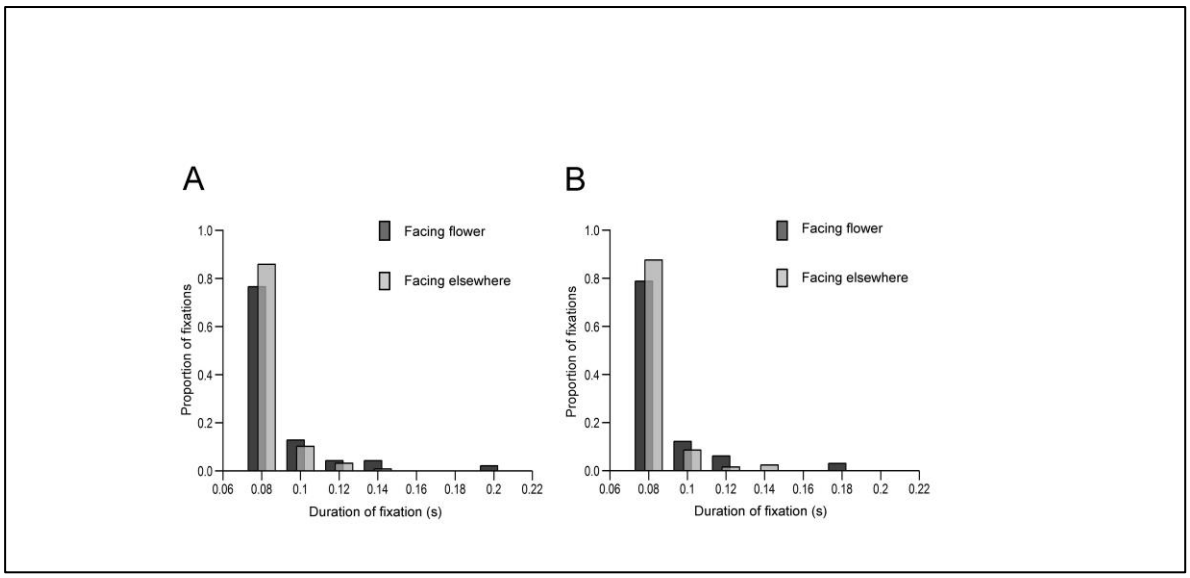


Figure 4.8. Duration of fixations in male and female learning flights. Normalised distributions of the duration of all fixations in (A) males (n=175 fixations, N=24 males) and (B) workers (n=162 fixations, N=14 workers). Male and female fixations are partitioned into those in which bees faced the flower (ϕ=0±20 deg; males n=47 fixations, workers n=33 fixations) or did not face the flower (φ<−20 deg or φ>20 deg).

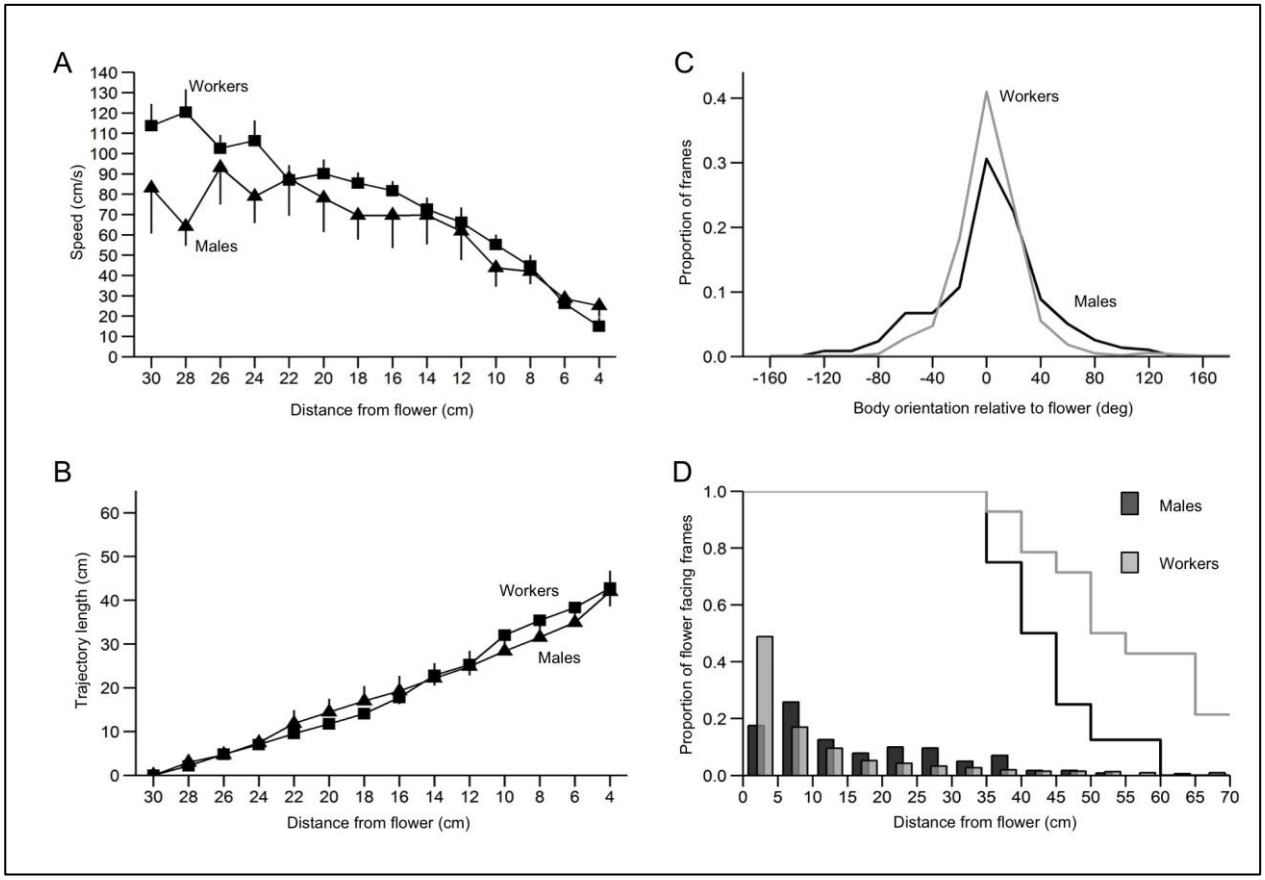


Figure 4.9. Comparison of male and worker returns to the flower. (A) Flight speed plotted against distance from the flower (N=8 males, N=14 workers). (B) Cumulative trajectory length as a function of distance from the flower. (C) Frequency distribution of body orientation relative to the flower (males, N=595 frames, workers N=1255). (D) Relative frequency of flower facing (ϕ=0±20 deg) plotted against distance from the flower (males, N=341 frames, workers N=811 frames). The lines above the histograms show the proportion of flights at each distance that are at least as long as that distance (N=8 males, N=14 workers).

The modal duration of these fixations was 80 ms in males and females, both when the bees fixated the flower within ±20 deg and when they looked elsewhere (Figure 4.8). In both sexes, the distribution of fixations peaked when bees faced the flower (Figure 4.7C), but workers spent slightly more of the flight facing the flower than did males. Thus, males faced the flower in 11.03±1.74% (mean±s.e.m.) of all frames and females in 14.42±2.42% of all frames (Mann– Whitney U-test, U=125.5, Z=1.29, P=0.20). In both males and females, fixations of the flower occurred most often when bees were close to the flower, within 5–10 cm of its centre (Figure 4.7D). The flights of males and females leaving the flower are thus quite comparable.

4.3.3 Returns to flowers

On several occasions, males were recorded when they flew back to revisit the flower (N=8, Figure 4.2E). These returns resembled those of workers. Like workers returning to the nest (Philippides et al., 2013), the male in Figure 4.2E faced the flower at the turning points of its zigzag approach. The flight speeds of males and workers dropped at about the same rate during the bee's approach to the flower (Figure 4.9A). Likewise, the path lengths of the bee's approach to the flower from 30 cm were similar in the two sexes (Figure 4.9B). Males faced the flower less often than workers, as seen in a slightly lower peak in the distribution of facing positions (Figure 4.9C). Workers faced the flower mostly when they were close to it, but males faced the flower over a broader range of distances (Figure 4.9D).

4.4 Discussion

Our results show that male and female bumblebees perform flights of a similar structure when leaving flowers, suggesting that males, like workers, perform learning flights when

leaving flowers. Although some males, like workers, returned to the flower after their learning flight, we do not know what males have memorised about the flower and its location during the flight. Male bumblebees have recently been found to learn the colours of artificial flowers and approach the rewarded colour while avoiding unrewarded colours (Wolf and Chittka, 2016), but it is unknown whether colour was memorised during a learning flight. The essence of this flight is that it contains periods of directed looking that are presumably coupled to the activation of learning. Thus, the patterns of fixation of a flower by males and females during learning flights (Figure 4.7) suggest that both sexes learn something about it – a conclusion that is reinforced by both males and workers facing the flower during their returns (Figure 4.9).

Bumblebee workers forage on a diversity of flowers in different locations, and it may be that learning the colour, shape and odour of a good species is just as or even more important than knowing a flower's exact location within a patch (Heinrich, 1979), though bumblebees can be faithful to the location of a patch, even when the flower species in the patch changes (Ogilvie and Thomson, 2016), indicating some locational learning of flowers. Honeybees can learn the details of a flower's appearance on both arrival and departure (Lehrer, 1993), but they seem to learn location in terms of a flower's proximity to other objects only on departure flights (Lehrer and Collett, 1994). It is unknown whether bumblebees are similar in this respect and whether males differ from females in their learning capacities.

Learning flights can be elicited by several factors such as drinking nectar or leaving a nest. They are also modulated by a variety of factors, like drinking time and sucrose concentration, enclosure in a hive or nest for protracted periods, and difficulty in finding a goal (e.g. Wagner, 1907; Wei et al., 2002; Wei and Dyer, 2009). The duration of a learning flight seems to be correlated with the significance of the place that the bee leaves. Thus, when they leave their nest, workers perform much longer flights than they leave a flower (Figure 4.6). It is

still unclear whether bees learn the same things when viewing flowers or the surroundings of the nest entrance.

Males of different bee species have a variety of mating strategies (Goulson, 2010; Paxton, 2005). In bumblebees, the commonest is that of *B. terrestris*, which patrols areas along routes. Other species are territorial and wait, hovering or perched, near a prominent visual object to pounce upon potential passing mates. Might males learn their visually specified hovering positions, as do patrolling halictine bees (Barrows, 1976)? Honeybee drones are quite different from bumblebees. They make excursions from their nest to drone aggregation sites to find and mate with queens (Galindo-Cardona et al., 2015; Loper et al., 1992; Witherell, 1971). When unsuccessful, they return to the nest for feeding, as do some male carpenter bees (Leys, 2000; Wittmann and Scholz, 1989). Does the occurrence of learning flights of honeybee and carpenter bee males also fit with their life style: learning flights when leaving the hive (to which they return for sustenance), instead of at flowers (which they do not visit)?

Chapter 5

General discussion

In this thesis, I explored how bumblebees vary their learning flights depending on the conditions in which they perform them. I have examined: 1) the relatively small differences that occur in learning flights from the nest and from an artificial flower; 2) how the flights from a flower vary when the flower delivers different rewards; 3) the marked similarities between flights from workers and males leaving a flower. Taken together, these results suggest that bumblebees have a standard template for performing this behaviour that however varies slightly to accommodate different circumstances and functions.

Finding similarities in shapes and durations between the flights of males and workers at the flower is convenient in reducing a methodological concern that what workers learnt at the nest, where the scene was made to be as similar as possible to that at the flower, might in some way have influenced their learning flights from the flower. Such an influence is impossible in males, since they generate learning flights when leaving flowers but learn nothing about the nest. Therefore, I can, here, be confident that the shorter learning flights of the workers at the flower is not an artefact of our protocol.

Indeed, the most obvious differences between the workers' flights from the nest and the flower are the differences in the duration of the flights and the way in which duration reduces over successive flights. The bumblebees' learning flights were about twice as long when leaving their nest than when they left a flower and the rate of decline over successive foraging trips was more rapid at the flower location. This difference suggests, for functional reasons given in Chapter 2 and 3, that bees invest more effort in learning about their nest than a flower. This suggestion is reinforced by finding that the length of flights from a flower

correlates with the sucrose concentration there. Both sets of results argue that the length of learning flights is related to the value that bees place on the site. However, if the notion of value is quantifiable in the case of various sugar concentrations offered by several flowers, it is more subjective when comparing the value of a flower with the value of the nest. The question follows to know how do the bees perceive the value of a location? When visiting a flower patch, would the bee consider each flower individually and perform a learning flight when leaving each of them for the next one? Or would the bee consider the entire patch as a valuable location and perform a longer learning flight at the scale of the flower patch itself? In any case, and surprisingly, the duration of the learning flight does not influence the accuracy of the bees' spatial memory. Indeed, I could not show any evidence for better learning at the nest than the flower either in the properties of their return flights to the two locations or in the precision of their performance during tests.

The question arises why a bee would invest more in learning a location if it does not improve its accuracy when returning to it. The answer to this question may come from the only difference seen between the bees' searches for their nest and for the flower: their persistence in locating the two goals during the unrewarded tests. The greater persistence at the nest could be related to memory strength. I can formulate the hypothesis that a mechanistic process (see discussion of Chapter 2) may exist that would increase a bee's persistence to search for a specific location proportionally to the time the insect spent performing a learning flight at this site. This would prove to be an adaptive mechanism because at a flower the bee has a choice to give up the search and look for another flower or patch, whereas with the nest there is only one adaptive solution, to temporarily give up but returning later. Indeed, in the wild, there is no evidence that bumblebee workers disperse easily into foreign nests, although in greenhouse settings, where colonies are kept in close vicinity and could be also closely related due to commercial breeding, drifting of workers has been reported (Birmingham and Winston,

2004). However, different to honeybees, bumblebees can survive outside the nest for a period of time. Foragers frequently stay outside the nest overnight and can persistently return to the nest over several days (N. Hempel de Ibarra personal communication, personal observations in the wild and the greenhouse).

Alternatively, the greater persistence at the nest could just be a consequence of the bees' motivational states at the two goals - a tendency to search harder for a nest than a flower (Chapter 2). To examine these two hypotheses, one could design an experiment to compare the search persistence of two groups of bumblebees. Because unsuccessful attempts to locate the nest during return flights increase the duration of the learning flights performed during the following departures, one could artificially induce longer learning flights in the first group of bumblebees by hiding the nest entrance when they return to their nest. The second group should be allowed to enter their nest freely during their returns. After a sequence of 3 to 4 foraging trips, both groups would be tested with the nest entrance hidden and their search persistence would be compared. According to the first hypothesis, because the bees from the first group would have invested a longer cumulated time in the successive learning flights, I would predict that their persistence greater than the one of the bees from the second group. Alternatively, if the second hypothesis is correct, the two group of bees would be in the same motivational state and therefore their search persistence would not be different.

However, other divergent characteristics of the learning flights at the nest and the flower may point toward a different but non-excusive explanation. Both during learning and return flights, bees tend to look at the area around the flower less than they scan around the nest. More scanning of the area around the nest may be an adaptation to an inconspicuous nest hole that is often hidden in low vegetation. If the nest hole is not directly visible in the wild, an approaching bee may well overshoot it and must be able to then recognise the hole's location from another direction. The bee may as well need to confirm that it pinpointed the proper

location before landing in the grass and search for the entrance by matching views in multiple directions. The fact that the bees also scan more the area around the nest than around the flower during their approaches supports this hypothesis.

If the area covered by the learning flights at the flower is smaller than at the nest, what do the bees learn when they depart from the flower? Flowers are by nature very conspicuous, thus bumblebees may only need to learn a very restricted set of views of the panorama during their departure from them to be able to return to their location. Because these rare views are directly oriented toward the nearest set of landmarks (see Figure 2.13A), they may well be enough to accurately pinpoint the flower location even when it was hidden during the tests. If this hypothesis is correct, the bees' accuracy when searching for the hidden flower would be more affected by a change in the characteristics of the nearest landmark (such as its size) than would be their accuracy in their search for the hidden nest where they could rely on numerous snapshots of the panorama memorised from multiple directions. This would represent a simple yet enlightening experiment to conduct in the future.

But flower location is only part of what the bees learn about food sources. Equally or more important is the ability to recognise the flower in other locations. A simple experiment could be designed to distinguish what in the learning flights performed at a flower is used to learn the characteristics of the flower itself and what is used to learn its location. A group of bees could be trained multiple times to a flower in one location then would be placed on the same flower at a new location. At the same time, a second group of bees would be placed on a different flower during their training and on a flower identical to the one of the first group at a new location. Would the bees' learning flights on a familiar flower at the new location differ from these of the bees that must learn both a new location and a new flower?

Another experimental protocol could reveal whether the learning of a flower's location or characteristics is prioritised by the bumblebees during their learning flights. Indeed, the

flower in my experiments was radially symmetric, but it might be insightful to examine whether learning flights would be affected if flowers were designed to be conspicuous from a narrow range of directions opposite to the directions of the nearest landmarks. Would the bees maintain a learning flight similar to the ones presented in Chapter 2, mostly oriented toward the landmarks? Or would the departure direction on learning flights adjust to give the bees the most informative view about the flower's characteristics and would bees then return facing the flower in the same direction? This experiment would help show that learning the appearance of flowers is a crucial part of flower learning flights (cf. Lehrer, 1993).

Pushing this idea further, how would the bees vary their learning flights if the flower was almost inconspicuous? Would the bees accentuate their attention toward the landmarks surrounding the scene and toward the panorama in an attempt to better learn the location? Or would they try to learn the very little details they could perceive of the flower?

To analyse and compare the results of the experiments proposed above, a useful indicator of the bees' attention to a specific object of the visual scene is the presence of fixations. In the thesis I have concentrated on fixations because they are likely to be those moments when bees are activating their learning circuits. It should be noted briefly here that learning is likely to occur also at other times and include moving views in addition to the static views certainly acquired during fixations. The wasp, *Cereceris* (Zeil, 1993b) and honeybees (Lehrer and Collett, 1993) learn the proximity of different visual features from a goal, presumably through motion parallax. And on their first few returns they search for the goal at the parallax defined distance. Honeybees can learn to locate an inconspicuous feeder in artificial arena lined with random-checkerboard pattern and three cylinders with the same pattern. The bees seem to rely on detection of optic flow cues for orienting in this space and adjusting their search for the feeder (Dittmar et al., 2010). However, the presence of fixations at the end of each arc of the learning flights of the wasp *Vespula vulgaris* and the fact that the insect assumed orientations

identical to these of the fixations during its return (Collett, 1995) prove that static views are certainly involved in the spatial learning process of the Hymenopterans (see also Chapter 1). It is significant that the number and rate of fixations of the goal during learning flights of bumblebees do correlate with the bees' valuation of the place that it learns. For example, bumblebees perform more fixations toward the nest than toward a flower. And their fixations are directed less precisely toward a flower with a low reward than with a high reward, perhaps indicating poorer learning of the flower's visual features.

Moreover, fixations are not only toward the goals. Bumblebees also fixated the cylinders that were placed near their nest or a flower. These cylinder fixations have several interesting properties. First and unlike goal fixations, cylinder fixations are only frequent during the bee's first learning and return flights. In later flights the cylinders are still essential for navigation (as seen in the bees' performance in tests), but the cylinders no longer need to be fixated, maybe indicating some yet un-investigated form of visual generalisation that does not require a retinotopic projection onto the eye of the bee in each of the subsequent learning flights. Alternatively, the presence of important numbers of cylinder fixations during the first learning flights at the nest and the flower and the decrease of the number of these fixations during the subsequent learning flights may indicate a particular learning strategy. The bees would prioritise the spatial learning relative to the nearest landmarks during their first learning flights at the two locations in order to ensure a higher chance to find the correct location on their returns. Then, during the subsequent learning flights at the nest, the bees would consolidate their learning in every direction of the panorama (including, but not especially, in direction of the nearest landmarks). At the flower, the subsequent learning flights would already stop being used to memorise the location (see Chapter 2).

Fortunately, I collected some experimental data still to be analysed that may shed light on this question. In this experiment, the position of the nest relative to a cylinder array was shifted

after three learning flights from the nest. This analysis will show whether bees fixate the cylinders again, on their fourth departure when they relearn the position of the nest. If the bees do not fixate the cylinders again, the result would support the hypothesis that the bees possess knowledge of the visual scene allowing them to generalise the position of the landmarks relative to the other elements of the environment and are be able to estimate the new position of the nest without fixating the cylinders. If the second hypothesis is true, the bees would fixate the cylinders again after their displacement.

The second interesting property of cylinder fixations is that different cylinders are fixated during learning flights from the nest and feeder flights and that the rates of fixation are higher during learning flights from the nest than from the feeder. This again emphasises that bees may learn more about the surroundings of a nest than a flower.

These last results show the importance of the fixation mechanism in the bumblebees' visual navigation process. The question follows whether other groups of flying insects also use similar fixations to visually learn their surroundings. Indeed, I have mentioned in Chapter 1 that Diptera such as Drosophila, hoverflies or blowflies are able to visually pinpoint a specific location (Collett and Land, 1975; Ofstad et al., 2011). Although they do not perform learning flights, it may be that these insects fixate their respective location of interest during their approach or departure from the sites. Pushing the idea further, walking insects may also use fixations when visually memorising a location. Nicholson et al. (1999) indicated in his study that the wood ant *Formica rufa* did regularly turn back toward its nest and more precisely toward the landmark placed next to it. More recently, Wystrach et al. (2014) have identified the presence of saccadic fixations of the panorama during similar turn back behaviour in the Australian desert ant *Melophorus bagoti*. Little is known about the presence of such fixations in non-Hymenopteran walking insects with the ability to visually navigate such as Blattodea (Rivault and Durier, 2004) or Orthoptera (Wessnitzer et al., 2008). For both walking and

flying insects, future studies should pay a particular attention to the possible presence of fixations enhancing visual spatial learning. The rapid development of high-speed camera and more accurate tracking software should help us to, in a near future, obtain the answers to the questions asked above.

Overall, the importance of the fixation reminds us that the bees are primarily visual animals. However, in the Chapter 1, I have mentioned that insects are capable to use two other strategies to navigate. Other groups of Hymenopterans, such as the ants, have been shown to use odour based navigations (Passera, 1984). In addition, Heinrich (1979) suggested that the smell of the flower could attract the bumblebees. In my experiments, I focussed of the visual learning ability of the bumblebees and made sure that my protocol did not involve odour cues, for example by using odourless sucrose and by cleaning regularly the flowers. Therefore, the question arises to know whether bumblebees would learn and use the scent present on a location to navigate more precisely. As the structure of the learning flights appears to be composed of both fixed and flexible components, how would the learning flights be different if the flowers were scented? Indeed, honeybees have been shown to use multisensory information, with the exposure to a familiar odour helping them to recall visual navigation information (Reinhard et al., 2004). Therefore, it is possible that bumblebees may sometimes have to compromise between the acquisition of the necessary visual information with the ability to perceive and learn the odour surrounding a location.

In general, the experiments presented in this thesis tell us how much we can learn from the detailed analysis of behaviour, but more experiments are needed to disentangle correlations from causes. Nevertheless, this work throws up numerous hypotheses and can be continued to test some of them. Overall, I hope to have added strong evidence that insect learning flights are highly adaptive behaviours. Even though learning flights are routed in a set of fixed motor

routines, insects display an impressive degree of flexibility that allows them to respond in appropriate ways to the constantly varying and unpredictable conditions in their environment.

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